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Research Article

Enrichment and Identification of Lignin–Carbohydrate Complexes in Softwood Extract

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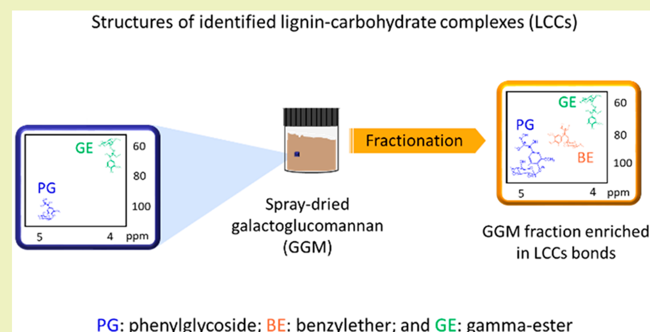
ABSTRACT: Lignin–carbohydrate complexes (LCCs) are hybrid structures containing covalently linked moieties of lignin and carbohydrates. The structure and behavior of LCCs affect both industrial processes and practical applications of lignocellulosic biomass. However, the identification of phenylglycoside, benzylether, and gamma (γ)-ester LCC bonds in lignocellulosic biomass is limited due to their relatively low abundance compared to plain carbohydrate and lignin structures. Herein, we enriched the LCC bonds in softwood galactoglucomannan (GGM)-rich extract fractionated by (1) a solvent (ethanol), (2) enzymes, and (3) physical techniques. Two-dimensional nuclear magnetic resonance (NMR) spectroscopy analysis was used to identify the LCC bonds.



Phenylglycoside and benzylether bonds were concentrated in the ethanol-soluble GGM fractions. A benzylether bond was concentrated into GGM fractions containing larger molecules (>500 Da) through physical techniques. The γ-ester bond was identified in all studied GGM fractions, which is explained by its stability and possible presence in residual xylan. In summary, we demonstrated the potential of the suggested techniques to enrich LCC bonds in softwood extract and improve LCC identification. Such techniques may also enable further studies on the structure and functionality of LCC bonds and open new prospects in the engineering of biomolecules.

KEYWORDS: *Antisolvent separation, Galactoglucomannan (GGM), Nanofiltration, Nuclear magnetic resonance (NMR) spectroscopy, Semi-simultaneous enzymatic hydrolysis (SSEH), Spruce wood, Ultracentrifugation*

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INTRODUCTION

Lignocellulosic biomass is a renewable and abundant source of natural biomolecules, widely distributed worldwide and the utilization of which is still expanding.[1] Sustainable alternatives from natural biomolecules are continually being developed to substitute products traditionally produced from fossil sources.[2] Similarly, more sustainable conditions for lignocellulosic biomass deconstruction have been implemented in biorefinery operations. This includes, for example, the use of hot water extraction, where acidic compounds from the lignocellulosic biomass perform autocatalysis.[2] In such extraction, the acetyl groups, involved in acetylation of hemicelluloses, such as softwood galactoglucomannan (GGM), act as catalytic agents.[3] Given that only water and no other chemicals/solvents are needed in hot water extraction, the substantial amounts of hemicelluloses and lignin in water extract are easily recovered.[2,4] The functionality and applicability of such extracts are known to depend on their chemical composition and purity.[1] For certain applications, such as cellulose dissolving pulp,[5] lignin carbon fibers,[6] and hemicellulose derivatization,[7] pure lignocellulosic fractions improve the quality of the end product. The existence of physical and chemical interactions between lignocellulosic components, however, is an unresolved limiting factor impacting the selective separation of pure moieties.[8−10]

Cellulose, hemicelluloses, and lignin, the main components in the plant cell wall, have unique chemical structures, properties, and functionality.[11] In lignocellulosic extracts, hybrid structures formed by covalently linked lignin and carbohydrates, so-called lignin–carbohydrate complexes (LCCs), have also been observed.[8,10,12,13] The copreservation of lignin and carbohydrate moieties may improve certain functionalities; for example, expression of antiviral, antioxidant, and antitumoral activities was attributed to some lignocellulosic extracts.[14,15] On the other hand, LCC structures are highly recalcitrant and prevent efficient lignocellulosic biomass deconstruction, likely due to the stability of certain LCC bonds.[10] The role of LCC bonds in the applicability of lignocellulosic extracts remains unknown, however. Only

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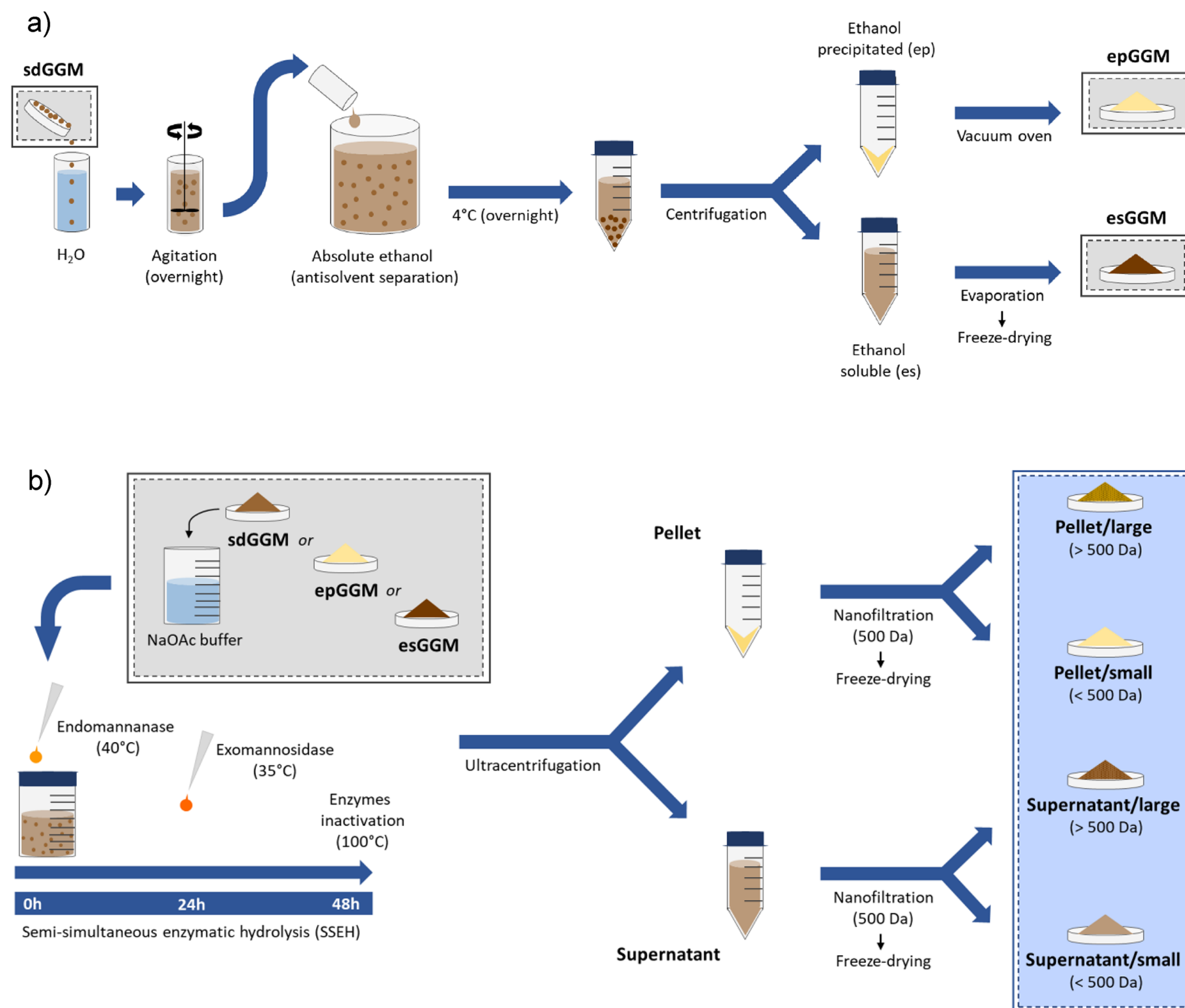
Scheme 1. (a) Flowchart of Preparation of Ethanol-Precipitated (epGGM) and Ethanol-Soluble (esGGM) Samples from Spray-Dried Galactoglucomannan (sdGGM) Sample Using Antisolvent Separation in Ethanol/Water System. (b) Fractionation of sdGGM, epGGM, and esGGM through Sequence of SSEH, ultracentrifugation, and nanofiltration.[a]



[a]Gray boxes show the three GGM samples used as starting materials, and the blue box shows the four different fractions obtained for each sample.

recently, a study hypothesized that LCC bonds improve emulsion stabilization by a GGM-rich extract.[16] However, a thorough characterization of GGM extract to identify the different LCC bonds has not been reported previously.

Nuclear magnetic resonance (NMR) spectroscopy is, currently, the most powerful tool used in LCC bond identification. The two-dimensional heteronuclear single quantum coherence (HSQC) technique correlates carbon and hydrogen signals and enables the identification of the various LCC bonds, i.e., phenylglycoside, benzylether, and γ-ester.[17] The relative low frequency of LCC bonds compared to plain carbohydrate and lignin structures in lignocellulosic extracts, however, is a limiting factor to the LCC identification. Our hypothesis is that LCC identification can be improved by fractionation. Ethanol separation, for example, proved to be efficient in precipitating a pure polymeric GGM fraction from pressurized hot water spruce extract, keeping most of the oligosaccharides and lignin in the ethanol phase.[18] Enzymatic hydrolysis using a selective enzyme cocktail is also useful to remove carbohydrate decorations from lignocellulosic samples.[8,12,17] Moreover, a recent study proved the ability of simple centrifugal forces to fractionate softwood extract into both hemicellulose-rich and lignin-rich fractions.[19] Herein, our aim was to combine various techniques and develop an analytical fractionation method to enrich LCC bonds in spruce GGM-rich extracts and improve LCC identification. For this we used (1) solvent separation (ethanol), (2) enzymes (endomannanase and exomannosidase), and (3) physical techniques (ultracentrifugation and nanofiltration). The solvent separation and physical techniques enabled the enrichment of LCC bonds in various GGM fractions, and the identification was achieved by HSQC spectra. The results facilitate understanding the role that LCCs play in biobased materials and promote the design of biorefinery processes to recover biomolecules with desired structures.

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■ MATERIAL AND METHODS

Materials. Spruce extract rich in galactoglucomannan (GGM) was prepared by a pressurized hot water flow-through extraction (PHWE) as described by Kilpeläinen et al.[4] GGM was recovered from the water extract by spray-drying (sdGGM).

Methods Applied for GGM Samples Preparing and Fractionating. *Preparation of GGM Samples Using Antisolvent Separation.* The sdGGM was used in the preparation of ethanol-soluble (esGGM) and ethanol-precipitated (epGGM) samples (Scheme 1a). The sdGGM was suspended in Milli-Q-water to a ratio of 8% solids, and the suspension was left agitating overnight at room temperature. Next, the suspension was slowly added to absolute ethanol to a suspension:ethanol concentration of 1:8, mixed for 10 min, and cooled at 4 °C overnight.[20] The precipitate was separated by centrifugation (20 °C, 10,000 rpm, 5 min), washed with absolute ethanol, and dried in a vacuum oven at 40 °C for at least 48 h to recover epGGM. The supernatant was concentrated using a rotary evaporator at 45−55 °C to remove the ethanol and lyophilized to recover esGGM. The yields were estimated gravimetrically.

Semi-Simultaneous Enzymatic Hydrolysis (SSEH). A semi-simultaneous enzymatic hydrolysis (SSEH) was performed to concentrate the LCC bonds in sdGGM, epGGM, and esGGM (Scheme 1b). This enzymatic hydrolysis was designed to reduce the molecular size of GGM through two mechanisms: cleaving the inner β-1,4 bond between either mannopyranosyl and glucopyranosyl or mannopyranosyl and mannopyranosyl units in the polysaccharide chain (endomannanase effect)[21] and eliminating the terminal nonreducing β-D-mannopyranosyl residues from the nonreducing end (exomannosidase effect).[22]

The amount of 1.0 g GGM (i.e., sdGGM, epGGM, or esGGM) was suspended in 100 mL of 0.1 M sodium acetate buffer (pH 5) (1% w/v) and supplemented with 5 U of endo-1,4-β-mannanase/g of GGM (Lot 00803, from *Aspergillus niger*, 42 U/mg from Megazyme). The suspension was incubated at 40 °C for 24 h.[23] Next, 5 U of exo-β-mannosidase/g of initial GGM (Lot 80501a, from *Cellulomonas fimi*, 23.5 U/mg from Megazyme) was added, and the suspension was incubated at 35 °C for an additional 24 h (no intermediate enzymatic inactivation was applied). This configuration allowed certain simultaneous activity of endomannanase in the hydrolytic conditions optimized for the exomannosidase. After completing the enzymatic hydrolysis time, both enzymes were inactivated by heating the suspension at 100 °C for 10 min. Hydrolysate was stored at 4 °C prior use. Alternatively, sdGGM, epGGM, and esGGM samples were incubated in water without enzyme supplementation (reference trials).

Ultracentrifugation. GGM hydrolysates from SSEH were fractionated by ultracentrifugation using an Optima L-90K Ultracentrifuge (Beckman Coulter, Inc., Brea, CA, USA) with a fixed angle rotor type 50.2 Ti (Beckman Coulter). Ultracentrifugation was performed at 40,000 rpm, for 1 h at 20 °C. The resulting supernatant and pellet (resuspended in Milli-Q-water) were collected and stored at 4 °C prior use (Scheme 1b).

Nanofiltration. A nanofiltration membrane with a 500−700 Da molecular weight cutoff (MWCO) (Synder Filtration, US, Kent) was used for the size fractionation of GGM samples. Both pellet and supernatant products from ultracentrifugation were filtered through the nanofiltration membrane (MWCO 500−700 Da) using a pressure of 1.5 bar nitrogen (Scheme 1b). Large molecules retained by the membranes (>500 Da) were washed at least four times with Milli-Q-water, collected, and freeze-dried. Small molecules poured through the membrane (<500 Da) were concentrated using a rotary evaporator at 45−55 °C and freeze-dried.

Chemical and Structural Assessments of GGM. *Determination of Carbohydrate Composition by Acid Methanolysis.* About 1 mg of sample (dry basis) was placed in a glass tube, suspended in 1 mL of 2 M hydrochloric acid in dry methanol, and flushed with nitrogen. Acid hydrolysis was performed at 100 °C for 5 h. Then, the suspension was neutralized with 200 μL of pyridine, cooled, and dried under a nitrogen flow. Next, 1 mL of 2 M trifluoroacetic acid was added to the sample, and the hydrolysis was carried out at 120 °C for 1 h. Then, the suspension was dried under a nitrogen flow. The sample was redissolved in Milli-Q-water (1 mL) and filtered through chromacol (0.45 μm) filters to the vials.[24,25] Hydrolysate was analyzed by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) using an ICS-3000 system (Dionex) equipped with a CarboPac PA1 column (4 × 250 mm, Dionex). The calibration curve was prepared using glucose, xylose, mannose, galactose, arabinose, rhamnose, fucose, glucuronic acid, and galacturonic acid (Sigma, Germany). This analysis was performed in three replicates.

Determination of Lignin Content. About 5 mg of lyophilized GGM samples was dissolved in 5 mL of 25% acetyl bromide in glacial acetic acid and 0.1 mL perchloric acid solution and heated at 70 °C under gentle stirring for 30 min. Next, the solution was cooled in an ice bath containing a small amount of ethanol, and 5 mL of cold 2 M sodium hydroxide (4 °C) and 12.5 mL of glacial acetic acid were added. The brominated products were analyzed by UV/vis spectrophotometer at 280 nm (Shimadzu UV2550). The extinction coefficient of the acetobrominated sample was determined for spruce (23.1 L g^{-1} cm^{-1}), using its lignin content (29.3%) obtained by Klason lignin analysis (T 222 om-02).[26,27] Acetobromination analysis was performed in duplicate.

Acetyl Content and Degree of Acetylation (DS_{Ac}). The acetyl content of GGM samples was determined by high-performance liquid chromatography (HPLC) after saponification. For this, 7 mg of sample (dry basis) was suspended in 300 μL of Milli-Q water, 1.2 mL of 0.8 M sodium hydroxide, and 10 μL of 1 M propionic acid (internal standard). The solution was heated at 60 °C overnight with agitation. Then, the solution was neutralized by 37% hydrochloric acid and filtered through chromacol (0.45 μm) filters to HPLC vials.[28] Glacial acetic acid was used for standards preparation. The analysis was performed in duplicate.

The degree of acetylation (DS_{Ac}) of GGM was determined from results of the acetyl content according to eq 1[29]

$$DS_{Ac} = \frac{Y \times \%\text{acetyl}}{(M_{\text{acetyl}} \times 100) - (M_{\text{acetyl}} - 1) \times \%\text{acetyl}} \quad (1)$$

where DS_{Ac} is the degree of acetylation of GGM samples, Y is the molecular weight of the anhydrous sugar in the GGM backbone (162 g/mol), % acetyl is the acetyl content determined by HPLC, and M_{acetyl} is the molecular weight of acetyl groups (43 g/mol).

Molar Mass Distribution. A 4 mg/mL solution was prepared by direct dissolution of GGM samples in SEC eluent (0.5% LiBr/DMSO) at 60 °C for 16 h and under constant agitation. Then, samples were filtered through chromacol (0.45 μm) filters and analyzed by size-exclusion chromatography (SEC) (SECcurity 1260, Polymer Standard Services, Mainz, Germany). A SEC instrument, coupled in series to a multiple-angle laser light scattering detector (MALLS; BIC-MwA7000, Brookhaven Instrument Corp., New York) and a refractive index detector (SECcurity 1260, Polymer Standard Services, Mainz, Germany) was used for the molar mass distribution assessment. A calibration curve was prepared using pullulan standards of known molar masses (Polymer Standards Services-PSS, Mainz, Germany). Samples were separated by GRAM columns (Polymer Standard Services, Mainz, Germany) where a precolumn (50 × 8 mm, 10 μm particle size), a 100 Å column, and a 10,000 Å column (300 × 8 mm, 10 μm particle size) were connected in series. Flow was 0.5 mL/min, and the column oven was at 60 °C, with the RI detector at 40 °C. The analysis was performed in duplicate.

Fourier Transform Infrared (FTIR) Spectroscopy. FTIR equipment (PerkinElmer FTIR spectrometer equipped with a Universal attenuated total reflection (ATR) sampling accessory) was used to collect spectra from dry GGM samples at room temperature. The parameters used were 4000−600 cm^{-1} wavelength, 16 scans at a resolution of 4 cm^{-1}, and intervals of 1 cm^{-1}. The resulting curves were baseline corrected and normalized (ref at 1600 cm^{-1}).

Nuclear Magnetic Resonance (NMR) Spectroscopy. A GGM sample of 25−40 mg was suspended in 700 μL of dimethyl sulfoxide-d_6 (DMSO-d_6) overnight at room temperature and with constant

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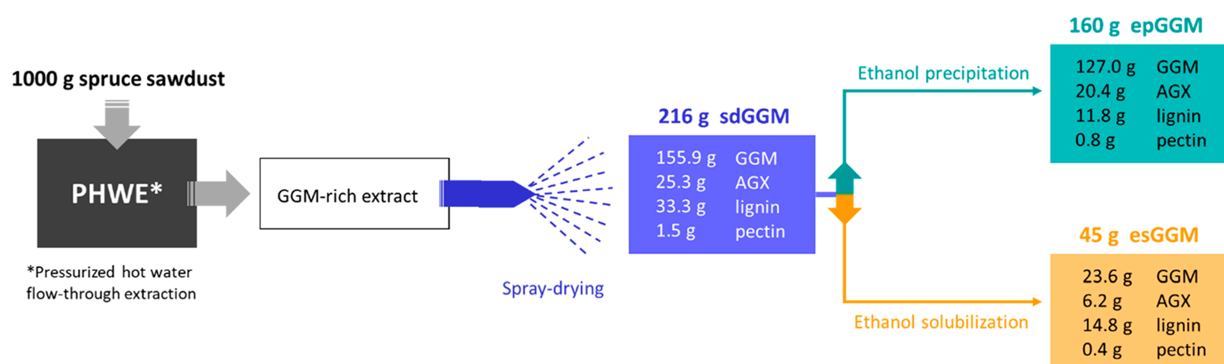

Figure 1. Mass balance and galactoglucomannan (GGM), arabinoglucuronoxylan (AGX), lignin, and pectin compositions of sdGGM, epGGM, and esGGM samples based on 1000 g of spruce raw sawdust.

stirring. All NMR measurements were collected using a Bruker Advance 850 MHz III high-definition spectrometer equipped with a cryoprobe (Bruker Corp., MA). Two-dimensional heteronuclear single quantum coherence spectroscopy (HSQC) experiments were carried out with Bruker pulse program hsqcedetgpsisp.2, size of FID of 2048, number of dummy scans of 32, and number of scans of 16. The spectra were processed using basic Fourier transformation, baseline correction in both dimensions, and phase correction. The distribution of acetyl groups in mannopyranosyl units was estimated by semi-quantitative analyses considering the methyl group in acetyl.

RESULTS AND DISCUSSION

Aspects of Preparation and Chemical Composition of GGM Samples. *Mass Balance.* To understand the composition of different GGM fractions and explain the selective enrichment of LCCs, the fractions were first characterized, and the mass balance after the antisolvent separation was considered. Spruce extract obtained from pressurized hot water flow-through extraction (PHWE) is a heterogeneous mixture containing about 24% of the original spruce wood.[4] This process efficiently dissolves more than 80% of hemicelluloses from spruce, together with a certain amount of residual lignin, extractives, and other minor compounds.[4,15] Interestingly, LCC structures are also dissolved, or formed, during this process.[10,15] Lignocellulosic compounds in water extract can be recovered through different processes, including spray-drying that enables an average of 90% recovery efficiency (data obtained from a similarly conducted spray-drying experiment, as yet unpublished). This suggests that a spray-dried GGM sample (sdGGM) contains about 21.6% of original spruce compounds, including galactoglucomannan (15.6%), arabinoglucuronoxylan (2.5%), lignin (3.3%), and pectin (0.2%). Herein, the various lignocellulosic compounds in sdGGM samples were separated by ethanol into two GGM populations (Figure 1). The purest GGM population was isolated through ethanol precipitation (epGGM)[18] and accounted for 16.0% on an original spruce sawdust basis. The more heterogeneous GGM population remained soluble in ethanol (esGGM) and only represented 4.5% of the original spruce sawdust. Interestingly, the various chemical and structural features of GGM samples correlated with the way in which GGM populations fractionated in ethanol. These observations were investigated and are discussed in the subsequent sections.

Factors Affecting Antisolvent Separation of GGM Populations in Ethanol. Our results confirmed that chemical composition and molar mass affected the dynamic (in)-solubility of GGM assemblies in ethanol. The results are depicted in Tables S1 and S2 and Figure S1 (Supporting Information) and are discussed herein based on differences in the chemical and structural characteristics of sdGGM, epGGM, and esGGM samples.

The sdGGM, which was used as the starting sample in this study, presented a heterogeneous chemical composition with 72.2% galactoglucomannan, 15.4% lignin, 11.7% arabinoglucuronoxylan, and 0.7% pectin. Its low molar mass (7.1 kDa) is likely explained by the partial hydrolysis of various polysaccharides during hot water extraction.[4] Indeed, the high dispersity index (4.3) and multimodal molar mass distribution (Supporting Information, Figure S1a) corroborated with the presence of different types of polysaccharides in the sample. A certain amount of acetyl groups (3.8%) was identified in sdGGM samples. It is noteworthy that small amounts of free acetic acid can be released during extraction and quantified together with acetyl groups.[18] Free acetic acid was not identified in sdGGM as confirmed by the absence of its typical band at 1706 cm^{-1} in FTIR spectra (Supporting Information, Figure S1b),[30] likely due to its elimination during sample drying. Since arabinoglucuronoxylan in spruce is not acetylated,[7] the degree of acetylation (DS_{Ac}) was calculated, taking into consideration acetylation only in the GGM backbone (eq 1). The DS_{Ac} of sdGGM (0.23) was close to the range reported in the literature for native GGM (0.25−0.33)[31] and in accordance with a partial deacetylation caused by the processes applied for sample preparation (i.e., hot water extraction and/or spray-drying).

The purer epGGM sample with 79.3% galactoglucomannan, 7.4% lignin, 12.7% arabinoglucuronoxylan, and 0.5% pectin was obtained from sdGGM by ethanol precipitation. epGGM had a higher molar mass (9.3 kDa) and lower dispersity index (3.6), in accordance with the literature.[20] The DS_{Ac} of the epGGM sample (0.27) was only slightly higher than that observed for the sdGGM (starting sample). Although traces of free acetic acid could be concentrated during antisolvent separation, this was not the case for epGGM. Free acetic acid is not expected to precipitate in ethanol, and for epGGM, it also would be eliminated during the freeze-drying process.[18,32] Moreover, the band for free acetic acid was not observed in FTIR spectra (Supporting Information, Figure S1b).[30]

esGGM was obtained by solubilization of the sdGGM sample in ethanol. Clearly, GGM populations assembled to more residues of other polysaccharides and lignin had a higher solubility in ethanol (52.4% galactoglucomannan, 32.9% lignin, 13.7% arabinoglucuronoxylan, and 1.0% pectin). Moreover, esGGM had a substantially lower molar mass (1.5 kDa) and a

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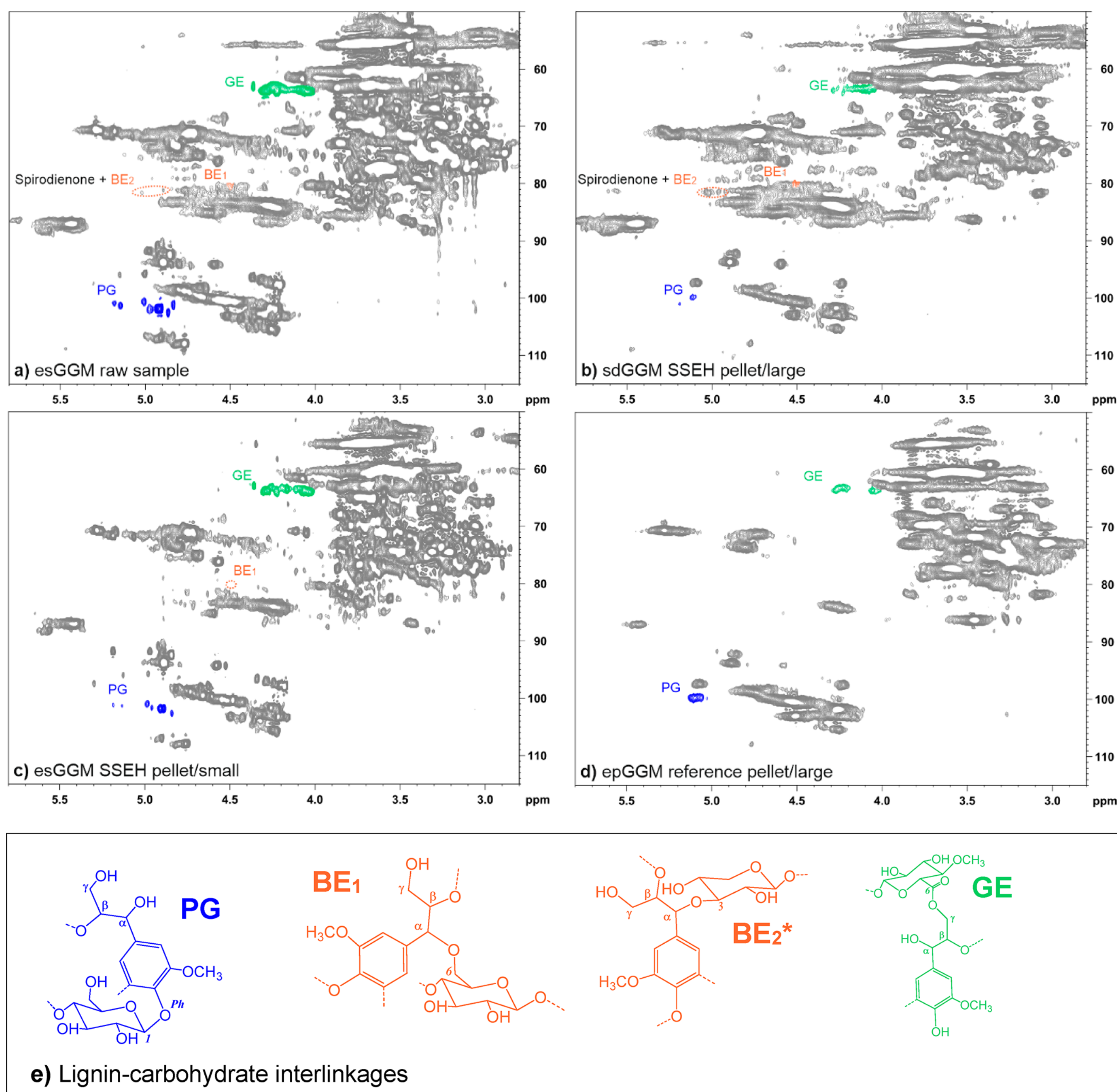

Figure 2. Evidence of presence of LCC interlinkages in (a) esGGM raw sample, (b) sdGGM SSEH pellet/large, (c) esGGM SSEH pellet/small, and (d) epGGM reference pellet/large. (e) Lignin−carbohydrate interlinkages for phenylglycoside (PG), benzylether (BE), and γ-ester (GE). Two structures of BE are possible: BE_1, linkage at C-6 in glucose, mannose, and galactose, or C-5 in arabinose; BE_2, linkage at C-2 or C-3 in glucose, mannose, galactose, xylose, and arabinose. *BE_2 was not identified in this study due to it overlapping with spirodienone signal. NMR spectra represented in this figure were chosen as examples to illustrate interesting aspects of the effect of the methods applied in the fractionation of LCC bonds and discussed herein. Other spectra obtained by this study can be found in the Supporting Information (Figures S2−S4).

more homogeneous molecular size distribution (2.0), supporting the fact that shorter polysaccharide populations were preferentially solubilized in ethanol. This observation is in accordance with the previous assertion of a greater solubility in ethanol of lignin and oligopolysaccharides, such as residual xylan.[18] Interestingly, no increased solubilization of pectin in ethanol was observed. The DS_{Ac} of esGGM (0.27) was similar to that observed for epGGM. Moreover, the acetylation distribution between C-2 and C-3 in mannosyl units (1.0:0.8, respectively) was similar among all three samples and slightly more frequent at the C-2 position. Despite the partial deacetylation that occurred during samples processing, the abundance of acetylation and its pattern in all three samples were consistent with those in the native GGM structure.[31] One possible explanation is that acetyl groups migrated to reestablish the native distribution pattern typical for GGM. This phenomenon was recently studied using mannose model compounds and indicated also that acetyl groups can migrate in both within the same mannose unit and across the glycosidic bond.[33]

Other aspects might also have some effects on antisolvent separation, such as acetylation spacing and/or clustering and

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the synergetic interactions of the various chemical compositions, acetylation degrees, and molar masses. However, the impact of such aspects and synergetic interactions could not be deduced from our study.

LCC Bonds in Solvent-Fractionated GGM Samples. HSQC results confirmed the potential of antisolvent separation to be used as an analytical method in the preparation of a GGM extract enriched in LCC bonds. The solubilization of GGM assemblies in ethanol enabled the visualization of signals for phenylglycoside, benzylether, and γ-ester by increasing the relative abundance of such bonds in the extract (Figure 2 and Supporting Information, Figures S2−S4). Other information about the main assignments for lignin, lignin substructures, lignin−carbohydrate bonds, and polysaccharides are shown in Tables S3, S4, S5, and S6, respectively (Supporting Information).

Phenylglycoside. Multiple contours in the area assigned to phenylglycoside (δC/δH 102−99/5.2−4.8 ppm)[10,12,34] were identified in sdGGM, esGGM, and epGGM. Comparatively, the esGGM sample clearly showed a greater abundance of contours at the phenylglycoside region (Figure 2a). This is explained by differences in the chemical structure of polysaccharides involved in the phenylglycoside bond, including carbohydrate composition and acetylation pattern.[12,17] Indeed, the phenylglycoside bond occurs between the phenolic hydroxyl in lignin and the hydroxyl at the reducing end of various types of carbohydrates.[8,10,12,34,35] The varied carbohydrate composition of sdGGM, esGGM, and epGGM (Supporting Information, Table S1) supported the appearance of multiple contours in the phenylglycoside region. Moreover, the great abundance of xylose, arabinose, and glucuronic acid in samples also suggested a possible involvement of residual xylan in the phenylglycoside linkage,[36] especially in esGGM samples. Furthemore, the lower molar mass of esGGM (1.5 kDa) likely increased the relative abundance of phenylglycoside bonds in the molecule, favoring their visualization. It is noteworthy that extractive compounds in spruce wood, such as stilbene and flavonoid, can also occur as glycosides.[37,38] The occurrence of hydroxystilbene-glycoside (δC/δH 108−100/6.5−5.9 ppm) and flavones (δC/δH 71.7/4.52; 83.0/5.00; 95.0/5.9; 96.0/5.9 ppm) in softwood were recently reported in the literature.[39,40] Although these compounds can be extracted by hot water extraction and concentrated by ethanol in esGGM, their typical signals distinguished from that used herein for the identification of phenylglycoside (δC/δH 102−99/5.2−4.8 ppm).

The presence of phenylglycoside in spruce in hot water extract, likely due to its solubilization from wood, is in accordance with the literature.[10] There are two speculative explanations for how phenylglycosides are formed. The first hypothesis relies on phenylglycoside formation in wood catalyzed by transglycosylating enzymes.[34,41,42] The second hypothesis relates to acid-catalyzed hydrolysis leading to acetal formation, which suggests that such reaction is more prone to occur at the proximity of acidic groups in the cell wall (e.g., glucuronic acid, galacturonic acid, and acetyl groups).[34,43]

Benzylether. Signals in the area attributed to the presence of benzylether (δC/δH 80.2/4.5 ppm)[13] were absent in sdGGM but were observed in esGGM, indicating the enrichment of benzylether bonds by ethanol solubilization (Figure 2a). In sdGGM, the benzylether bonds, likely present in very low frequency, had their signals partially masked by the high abundance of signals from lignin and carbohydrates. Ethanol separation caused an increase in lignin content in esGGM (32.9%), which was also accompanied by a substantial reduction in its molar mass (1.5 kDa). This increased the relative frequency of benzylether bonds in esGGM and the intensity of their signals, thus enabling visualization. The signal for benzylether in the secondary hydroxyl group (BE_2) (δC/δH 81−80/5.1−4.9 ppm), which is mainly associated with a xylan−ether bond, can be overlapped with spirodienone lignin signals.[17] The presence of spirodienone was confirmed in some GGM fractions (Supporting Information, Table S4);[44] thus, only benzylether in the primary hydroxyl group (BE_1) was tentatively assigned in this study. Signals for BE_1 had been identified in several lignocellulosic preparations at δC/δH 82−80/4.8−4.5 ppm,[10,12,17,34−36] but its occurrence between lignin and mannose in milled softwood lignin was only recently unequivocally confirmed using both 2D and 3D NMR techniques.[13] Accordingly, we only assigned HSQC correlation (δC/δH 80.2/4.5 ppm) to benzylether linkages involving C6-OH in mannan residues.[13] Benzylether is formed through a nucleophilic attack of hydroxyl groups (primary or secondary) from various carbohydrate residues to the electrophilic site of the quinone methide intermediate of lignin.[8,34,45]

γ-Ester. The γ-ester bond was identified in all three samples, sdGGM, epGGM, and esGGM (δC/δH 65.0−62.0/4.5−4.0 ppm),[17] with no clear effect of antisolvent separation on its fractionation, which is likely due to its high stability.[10] The γ-ester bond originates from the linkage of uronic acid moieties to the γ-position of lignin.[8,46] First, an α-ester bond intermediate is formed through a mechanism similar to that described previously for benzylether formation. α-Ester is less thermodynamically favorable than γ-ester, leading to ester-bond migration to the γ-position via a transesterification reaction.[34,45,47,48] This migration can occur under neutral and acidic conditions and, potentially, during isolation processes under those conditions.[10,47,48] As expected, GGM samples exhibited no signals for ester at the α-position (δC/δH 77−75/6.2−6.0 ppm).[48]

At least part of γ-ester in the GGM samples is likely from the xylan−lignin linkage. The possible role of the lignin−carbohydrate ester bond in the retention of xylan and pectin during spruce hot water extraction was reported previously.[10] A study on the fractionation of spruce extract using physical methods recently confirmed the existence of a linear correlation between the content of lignin and uronic acid and lignin and xylose in fractions.[15] Hence, these authors also suggested the presence of xylan−lignin ester bonds in GGM extract, but no cross signals at δC/δH 65.0−62.0/4.5−4.0 ppm were identified, likely due the low frequency of ester bonds. Carboxylic acid groups in glucuronic acid are known sites of γ-ester bond formation between xylan and lignin.[36] We identified arabinoglucuronoxylan (AGX), the second most abundant hemicellulose in spruce wood, in the GGM samples (11.7%−13.7%), along with a certain amount of glucuronic acid (1.6%−2.7%). Even though the galacturonic acid from pectin can also be involved in γ-ester linkage in lignin,[36] only traces of galacturonic acid were observed in the samples (below 0.1%). A xylan−lignin ester bond, however, is not the only explanation for the presence of γ-ester in GGM fractions. As GGM is a noncharged polysaccharide, the occurrence of γ-ester in GGM samples may also be explained by the partial acetylation of lignin primary alcohol at the γ-position.[10] Although some lignins are naturally acetylated at the γ-position, this is not the case for spruce lignin.[49] Thus, the

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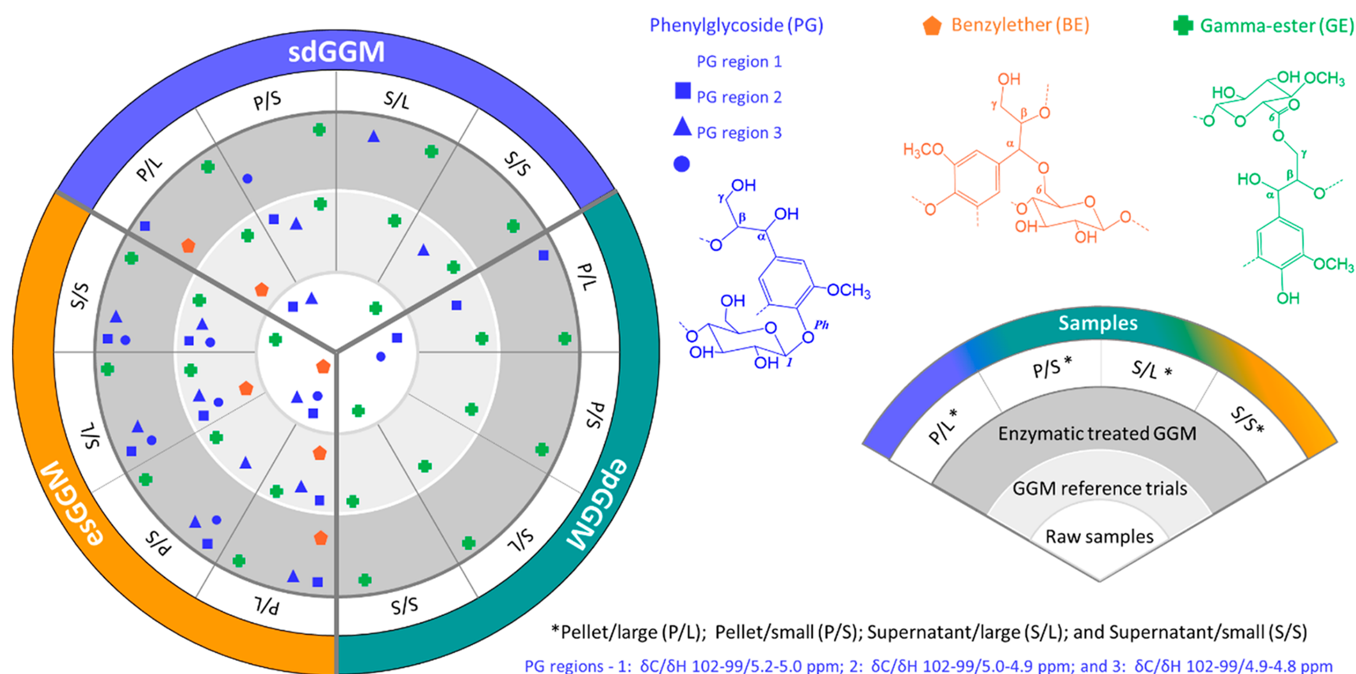

Figure 3. Summary of phenylglycoside, benzylether, and γ-ester identification in GGM samples and fractions assessed in HSQC spectra. The sdGGM, esGGM, and epGGM raw samples were fractionated by enzymatic treatment and ultracentrifugation/nanofiltration methods generating the fractions described in the figure (∗). The GGM reference trials refer to the samples obtained only by ultracentrifugation/nanofiltration methods, i.e., without enzymatic hydrolysis.

probable origin of this acetylation in lignin is the migration of the acetyl group from the secondary alcohol in carbohydrates to the primary alcohol in the lignin during the isolation process.[10]

Enzymatic and Physical Fractionation. sdGGM, epGGM, and esGGM were further fractionated by enzymatic treatment and physical methods (ultracentrifugation followed by nanofiltration) to achieve the enrichment of LCC bonds and facilitate their identification. Reference trials without enzymatic hydrolysis were also performed, and the fractionation was solely based on solubility (ultracentrifugation) and molecular size (nanofiltration). The following GGM fractions were obtained: pellet/large, pellet/small, supernatant/large, and supernatant/small, referring respectively to the ultracentrifugation and nanofiltration outcomes (Scheme 1b). Next, the results are discussed based on the various types of LCC bonds identified in the various fractions (Figure 3).

Phenylglycoside. The cross signal assigned to phenylglycoside (δC/δH 101.8−99.8/5.2−4.8 ppm) was identified in all esGGM fractions and most of the sdGGM fractions (Figure 2 and 3 and Supporting Information, Figures S2−S4) in accordance with the literature.[10,12,34] Surprisingly, phenylglycoside was substantially concentrated in epGGM pellet/large fractions (with and without enzymatic treatment) as indicated by the broader contour at δC/δH 99.8/5.1 ppm (Figure 2d and Supporting Information, Figure S4e) and disappearance of typical signals in other epGGM fractions (Figure S4, Supporting Information). Such results indicated that the phenylglycoside present in the epGGM sample was associated with large molecules unaffected by the enzymatic treatment. Thus, the physical techniques were more efficient in fractionating and enriching the phenylglycoside bonds in GGM fractions than the enzymatic treatment. Most of the GGM fractions exhibited more than one contour in the phenylglycoside region due to the participation of different carbohydrate moieties in the phenylglycoside bond,[12,17] which was most abundant in esGGM fractions. The enzymatic hydrolysis was expected to improve the LCC identification by removing carbohydrate decorations not involved in the lignin−carbohydrate linkage.[8,12,17] No such improvement was noticed herein, although changes in the mobility of LCC bonds among fractions were observed in response to GGM chain fragmentation. The enzymes supplemented in this study were not expected to hydrolyze the linkage between lignin and GGM due to their specificity toward the GGM chain. However, the cleavage of phenylglycoside by cellulolytic enzymes has been reported.[17] Therefore, the occurrence of a similar mechanism cleaving the linkage between mannose and lignin by mannolytic enzymes, to some extent, cannot be excluded, although still requires further investigation.

Benzylether. The signal in the region attributed to the occurrence of benzylether (δC/δH 80.2/4.5 ppm)[13] was only identified in sdGGM pellet/large, esGGM pellet/large, and esGGM supernatant/large fractions in reference trials and sdGGM pellet/large and esGGM pellet/large in enzymatic treated fractions (Figure 3). Moreover, benzylether signals were also overlapped by other signals from carbohydrates and lignin (Figure 2a and b and Supporting Information, Figures S2 and S3). Interestingly, benzylether signals absent in sdGGM became visible after physical fractionation in sdGGM pellet/large (with and without enzymatic treatment), confirming that fractionation increased the abundance of benzylether bonds by removing carbohydrate-soluble moieties.[19] This result confirmed the possibility of concentrating the benzylether structures in GGM assemblies using mild and inert physical methods. The literature had reported the use of a broader area for the benzylether assignment (i.e., δC/δH 82−80/4.8−4.5 ppm).[10,12,17,34,36] Using this approach, the benzylether bond would also be identified in the esGGM SSEH pellet/small fraction (Figure 2c). Even so, benzylether signals were still

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absent in all epGGM fractions, confirming the efficiency of ethanol in solubilizing LCC ether bonds. The enzymatic hydrolysis applied in this study did not further concentrate benzylether bonds in GGM fractions. Indeed, the presence of benzylether was identified in similar fractions, with or without enzymatic treatment. It is noteworthy that no signal at $\delta C/\delta H$ 80.2/4.5 ppm was visible after enzymatic hydrolysis for the esGGM supernatant/large fraction, supporting the hypothesis that GGM fragmentation affected LCC solubility and mobility during the physical fractionation. On the other hand, it is intriguing that benzylether bonds were only observed in large molecular-sized GGM populations (>500 Da). One possible explanation relies on the benzylether structure or, more specifically, on the abundance of available sites for benzylether linkage in carbohydrates. Carbohydrates have several hydroxyl groups available for ether bonds. This likely enables the linkage of more than one lignin residue to the same polysaccharide via ether bonds. The presence of various lignin residues bonded to a single polysaccharide would significantly increase the molecular size of the GGM assembly. During the enzymatic hydrolysis, however, the GGM chain is eventually hydrolyzed by enzymes, resulting in its molar mass reduction. This apparently did not occur in the benzylether-containing GGM fractions suggesting that benzylether, somehow, protected the polysaccharide against enzymatic attack.

γ-Ester. Contours assigned to CH_2-γ in γ-esters observed in the area of $\delta C/\delta H$ 65.0−62.0/4.5−4.0 ppm confirmed the presence of γ-ester in all GGM fractions (Figure 3).[48] The fractionation performed in this study appeared to have no effect on the distribution of γ-esters among GGM fractions (Figure 2 and Supporting Information, Figures S2−S4). Indeed, the great stability of γ-ester bonds and their wide occurrence in both large and small molecules has been reported.[10] As discussed earlier with reference to GGM samples, residual xylan likely plays a role in the presence of ester bonds in all fractions. According to the specificity of the enzymatic cocktail used in this study, enzymatic hydrolysis likely did not modify the xylan structure leading, hence, to the preservation of γ-ester bonds. In accordance with that reported in the literature,[48] no residual ester bonds at the α-position ($\delta C/\delta H$ 77−75/6.2−6.0 ppm) were identified in GGM fractions.

Remarkable Findings and Prospects. Despite the recognized recalcitrance of LCC structures,[10] LCCs have been associated with the expression of various functionalities, such as antiviral, antioxidant, and antitumoral activities.[14,15] Such functionalities, however, are more likely due to the copreservation of moieties of lignin and carbohydrates than to the LCC bonds themselves. Only recently, a study comparing the performance of epGGM and untreated GGM extract as emulsifiers indicated, for the first time, a possible functionality of LCC bonds.[16] Oil-in-water emulsion prepared using GGM extract, rich in lignin, presented better emulsion stabilization capacity than the more pure epGGM.[50] The presence of phenylglycoside and γ-ester bonds at the emulsion droplet interface was suggested. In this arrangement, LCC bonds would strategically connect the lignin residues at the oil droplet to carbohydrate tails in the water phase and, therefore, stabilize the emulsion.[16] Herein, we confirmed the presence and identified phenylglycoside and γ-ester bonds in sdGGM and epGGM. Additionally, we enriched phenylglycoside and benzylether bonds in certain sdGGM, esGGM, and epGGM fractions, which will enable better understanding of their roles in material applications. Specifically, the use of ultracentrifugation and nanofiltration enriched the LCC bonds. Recovering all lignocellulosic biomass fractions fits well in circular (bio)economy vision, by promoting a more sustainable utilization of lignocellulosic byproducts.[1] The developed analytical fractionation, enrichment, and identification of phenylglycoside, benzylether, and γ-ester in lignocellulosic materials will enable determination of their roles in emulsion stabilization as well as in other potential applications, as well as the ability to tailor and design the biomass treatment processes.

CONCLUSION

The combination of chemical (ethanol separation) and physical (ultracentrifugation/nanofiltration) techniques used in this study promoted the fractionation and enrichment of LCC bonds in certain GGM fractions, confirming the initial hypothesis. A phenylglycoside bond was preferentially solubilized by ethanol and thus enriched in the esGGM. The different carbohydrate residues involved in phenylglycoside bonds lead to the formation of various phenylglycoside structures, identified as multiple contours in HSQC spectra. Phenylglycoside LCC structures were modified by enzymatic hydrolysis and fractionated by physical methods. A benzylether bond was enriched and fractionated in GGM fractions by chemical and physical techniques, respectively. The γ-ester bonds were evenly distributed among GGM fractions, irrespective of the fractionation technique applied. This suggested that at least part of the γ-ester in the samples were due to the γ-ester bond between residual xylan lignin.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssuschemeng.0c03988.

Mass balance and chemical characterization, molar mass distribution spectra, FTIR spectra, assignment for bands in FTIR spectra, assignment for signals in NMR spectra, and NMR spectra (PDF)

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Author Contributions

The manuscript was written through the contributions of all authors. All authors approved the final version of the manuscript. D.M.d.C. performed all the experiments and wrote and edited the manuscript. K.S.M. and M.L. supervised the study. K.S.M., M.H.L., and M.L. revised the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

AGX, arabinoglucuronoxylan; epGGM, ethanol-precipitated galactoglucomannan; esGGM, ethanol-soluble galactoglucomannan; FTIR, Fourier transform infrared spectroscopy; GGM, galactoglucomannan; HMBC, ^{1}H-^{13}C heteronuclear multiple bond correlation; HSQC, heteronuclear single quantum coherence spectroscopy; LC, liquid chromatography with tandem mass spectrometry (LC-MS/MS); LCC, lignin–carbohydrate complex; MWCO, molecular weight cutoff; NMR, nuclear magnetic resonance spectroscopy; PHWE, pressurized hot water flow-through extraction; sdGGM, spray-dried galactoglucomannan; SSEH, semi-simultaneous enzymatic hydrolysis; γ-ester, gamma-ester

■ REFERENCES

(1) Mikkonen, K. S. Strategies for structuring diverse emulsion systems by using wood lignocellulose-derived stabilizers. *Green Chem.* **2020**, *22* (4), 1019–1037.

(2) Amidon, T. E.; Liu, S. Water-based woody biorefinery. *Biotechnol. Adv.* **2009**, *27* (5), 542–550.

(3) Garrote, G.; Domínguez, H.; Parajó, J. C. Mild autohydrolysis: an environmentally friendly technology for xylooligosaccharide production from wood. *J. Chem. Technol. Biotechnol.* **1999**, *74* (11), 1101–1109.

(4) Kilpeläinen, P. O.; Hautala, S. S.; Byman, O. O.; Tanner, L. J.; Korpinen, R. I.; Lillandt, M. K. J.; Pranovich, A. V.; Kitunen, V. H.; Willför, S. M.; Ilvesniemi, H. S. Pressurized hot water flow-through extraction system scale up from the laboratory to the pilot scale. *Green Chem.* **2014**, *16* (6), 3186–3194.

(5) Sixta, H.; Iakovlev, M.; Testova, L.; Roselli, A.; Hummel, M.; Borrega, M.; van Heiningen, A.; Froschauer, C.; Schottenberger, H. Novel concepts of dissolving pulp production. *Cellulose* **2013**, *20* (4), 1547–1561.

(6) Chatterjee, S.; Saito, T.; Rios, O.; Johs, A. Lignin based carbon materials for energy storage applications. In *Green Technologies for the Environment*; Obare, S. O., Luque, R., Eds.; ACS Symposium Series 1186; American Chemical Society: Washington DC, 2014; pp 203–218. DOI: 10.1021/bk-2014-1186.ch011.

(7) de Carvalho, D. M.; Berglund, J.; Marchand, C.; Lindström, M. E.; Vilaplana, F.; Sevastyanova, O. Improving the thermal stability of different types of xylan by acetylation. *Carbohydr. Polym.* **2019**, *220*, 132–140.

(8) Balakshin, M.; Capanema, E.; Berlin, A. Isolation and Analysis of Lignin–Carbohydrate Complexes Preparations with Traditional and Advanced Methods: A Review. In *Studies in Natural Products Chemistry*; Atta ur, R., Ed.; Elsevier: Amsterdam, 2014; Vol. *42*, pp 83–115. DOI: 10.1016/B978-0-444-63281-4.00004-5.

(9) Arnling Bååth, J.; Giummarella, N.; Klaubauf, S.; Lawoko, M.; Olsson, L. A glucuronoyl esterase from Acremonium alcalophilum cleaves native lignin-carbohydrate ester bonds. *FEBS Lett.* **2016**, *590* (16), 2611–2618.

(10) Giummarella, N.; Lawoko, M. Structural Insights on Recalcitrance during Hydrothermal Hemicellulose Extraction from Wood. *ACS Sustainable Chem. Eng.* **2017**, *5* (6), 5156–5165.

(11) Du, X.; Gellerstedt, G.; Li, J. Universal fractionation of lignin–carbohydrate complexes (LCCs) from lignocellulosic biomass: an example using spruce wood. *Plant J.* **2013**, *74* (2), 328–338.

(12) Du, X.; Pérez-Boada, M.; Fernández, C.; Rencoret, J.; del Río, J. C.; Jiménez-Barbero, J.; Li, J.; Gutiérrez, A.; Martínez, A. T. Analysis of lignin–carbohydrate and lignin–lignin linkages after hydrolase treatment of xylan–lignin, glucomannan–lignin and glucan–lignin complexes from spruce wood. *Planta* **2014**, *239* (5), 1079–1090.

(13) Nishimura, H.; Kamiya, A.; Nagata, T.; Katahira, M.; Watanabe, T. Direct evidence for α ether linkage between lignin and carbohydrates in wood cell walls. *Sci. Rep.* **2018**, *8* (1), 1–11.

(14) Sakagami, H.; Hashimoto, K.; Suzuki, F.; Ogiwara, T.; Satoh, K.; Ito, H.; Hatano, T.; Takashi, Y.; Fujisawa, S.-i. Molecular requirements of lignin–carbohydrate complexes for expression of unique biological activities. *Phytochemistry* **2005**, *66* (17), 2108–2120.

(15) Zhang, Y.; Wang, S.; Xu, W.; Cheng, F.; Pranovich, A.; Smeds, A.; Willför, S.; Xu, C. Valorization of lignin–carbohydrate complexes from hydrolysates of norway spruce: efficient separation, structural characterization, and antioxidant activity. *ACS Sustainable Chem. Eng.* **2019**, *7* (1), 1447–1456.

(16) Lehtonen, M.; Merinen, M.; Kilpeläinen, P. O.; Xu, C.; Willför, S. M.; Mikkonen, K. S. Phenolic residues in spruce galactoglucomannans improve stabilization of oil-in-water emulsions. *J. Colloid Interface Sci.* **2018**, *512*, 536–547.

(17) Balakshin, M.; Capanema, E.; Gracz, H.; Chang, H.-m.; Jameel, H. Quantification of lignin–carbohydrate linkages with high-resolution NMR spectroscopy. *Planta* **2011**, *233* (6), 1097–1110.

(18) Song, T.; Pranovich, A.; Holmbom, B. Separation of polymeric galactoglucomannans from hot-water extract of spruce wood. *Bioresour. Technol.* **2013**, *130*, 198–203.

(19) Valoppi, F.; Lahtinen, M. H.; Bhattarai, M.; Kirjoranta, S. J.; Juntti, V. K.; Peltonen, L. J.; Kilpeläinen, P. O.; Mikkonen, K. S. Centrifugal fractionation of softwood extracts improves the biorefinery workflow and yields functional emulsifiers. *Green Chem.* **2019**, *21* (17), 4691–4705.

(20) Mikkonen, K. S.; Merger, D.; Kilpeläinen, P.; Murtomäki, L.; Schmidt, U. S.; Wilhelm, M. Determination of physical emulsion stabilization mechanisms of wood hemicelluloses via rheological and interfacial characterization. *Soft Matter* **2016**, *12* (42), 8690–8700.

(21) Yeoman, C. J.; Han, Y.; Dodd, D.; Schroeder, C. M.; Mackie, R. I.; Cann, I. K. Thermostable Enzymes As Biocatalysts in the Biofuel Industry. In *Advances in Applied Microbiology*; Elsevier: San Diego, 2010; Vol. *70*, pp 1–55. DOI: 10.1016/S0065-2164(10)70001-0.

(22) Chauhan, P. S.; Gupta, N. Insight into microbial mannosidases: a review. *Crit. Rev. Biotechnol.* **2017**, *37* (2), 190–201.

(23) Parikka, K.; Leppänen, A.-S.; Xu, C.; Pitkänen, L.; Eronen, P.; Österberg, M.; Brumer, H.; Willför, S.; Tenkanen, M. Functional and anionic cellulose-interacting polymers by selective chemo-enzymatic carboxylation of galactose-containing polysaccharides. *Biomacromolecules* **2012**, *13* (8), 2418–2428.

(24) Bertaud, F.; Sundberg, A.; Holmbom, B. Evaluation of acid methanolysis for analysis of wood hemicelluloses and pectins. *Carbohydr. Polym.* **2002**, *48* (3), 319–324.

(25) Appeldoorn, M. M.; Kabel, M. A.; Van Eylen, D.; Gruppen, H.; Schols, H. A. Characterization of oligomeric xylan structures from corn fiber resistant to pretreatment and simultaneous saccharification and fermentation. *J. Agric. Food Chem.* **2010**, *58* (21), 11294–11301.

(26) Standard Test Methods. In *Acid-Insoluble Lignin in Wood and Pulp*; Tappi, Technical Association of the Pulp and Paper Industry: Atlanta, GA, 2006.

(27) Giummarella, N.; Zhang, L.; Henriksson, G.; Lawoko, M. Structural features of mildly fractionated lignin carbohydrate complexes (LCC) from spruce. *RSC Adv.* **2016**, *6* (48), 42120−42131.
(28) Bi, R.; Berglund, J.; Vilaplana, F.; McKee, L. S.; Henriksson, G. The degree of acetylation affects the microbial degradability of mannans. *Polym. Degrad. Stab.* **2016**, *133*, 36−46.
(29) Xu, C.; Leppänen, A.-S.; Eklund, P.; Holmlund, P.; Sjöholm, R.; Sundberg, K.; Willför, S. Acetylation and characterization of spruce (Picea abies) galactoglucomannans. *Carbohydr. Res.* **2010**, *345* (6), 810−816.
(30) Shamekhi, M. A.; Rabiee, A.; Mirzadeh, H.; Mahdavi, H.; Mohebbi-Kalhori, D.; Eslaminejad, M. B. Fabrication and characterization of hydrothermal cross-linked chitosan porous scaffolds for cartilage tissue engineering applications. *Mater. Sci. Eng., C* **2017**, *80*, 532−542.
(31) Sjöström, E. Wood Polysaccharides. In *Wood Chemistry*, Second ed.; Sjöström, E., Ed.; Academic Press: San Diego, 1993; pp 51−70.
(32) Furuike, T.; Komoto, D.; Hashimoto, H.; Tamura, H. Preparation of chitosan hydrogel and its solubility in organic acids. *Int. J. Biol. Macromol.* **2017**, *104*, 1620−1625.
(33) Lassfolk, R.; Rahkila, J.; Johansson, M. P.; Ekholm, F. S.; Wärnå, J.; Leino, R. Acetyl group migration across the saccharide units in oligomannoside model compound. *J. Am. Chem. Soc.* **2019**, *141* (4), 1646−1654.
(34) Giummarella, N.; Balakshin, M.; Koutaniemi, S.; Kärkönen, A.; Lawoko, M. Nativity of lignin carbohydrate bonds substantiated by biomimetic synthesis. *J. Exp. Bot.* **2019**, *70* (20), 5591−5601.
(35) Martínez-Abad, A.; Giummarella, N.; Lawoko, M.; Vilaplana, F. Differences in extractability under subcritical water reveal interconnected hemicellulose and lignin recalcitrance in birch hardwoods. *Green Chem.* **2018**, *20* (11), 2534−2546.
(36) Deshpande, R.; Giummarella, N.; Henriksson, G.; Germgård, U.; Sundvall, L.; Grundberg, H.; Lawoko, M. The reactivity of lignin carbohydrate complex (LCC) during manufacture of dissolving sulfite pulp from softwood. *Ind. Crops Prod.* **2018**, *115*, 315−322.
(37) Hammerbacher, A.; Ralph, S. G.; Bohlmann, J.; Fenning, T. M.; Gershenzon, J.; Schmidt, A. Biosynthesis of the major tetrahydroxystilbenes in spruce, astringin and isorhapontin, proceeds via resveratrol and is enhanced by fungal infection. *Plant Physiol.* **2011**, *157* (2), 876−890.
(38) Metsämuuronen, S.; Sirén, H. Bioactive phenolic compounds, metabolism and properties: a review on valuable chemical compounds in Scots pine and Norway spruce. *Phytochem. Rev.* **2019**, *18* (3), 623−664.
(39) Rencoret, J.; Neiva, D.; Marques, G.; Gutiérrez, A.; Kim, H.; Gominho, J.; Pereira, H.; Ralph, J.; del Río, J. C. Hydroxystilbene glucosides are incorporated into Norway spruce bark lignin. *Plant Physiol.* **2019**, *180* (3), 1310−1321.
(40) Faleva, A. V.; Kozhevnikov, A. Y.; Pokryshkin, S. A.; Falev, D. I.; Shestakov, S. L.; Popova, J. A. Structural characteristics of different softwood lignins according to 1D and 2D NMR spectroscopy. *J. Wood Chem. Technol.* **2020**, *40* (3), 178−189.
(41) Fry, S. C. Polysaccharide-modifying enzymes in the plant cell wall. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1995**, *46* (1), 497−520.
(42) Simmons, T. J.; Mohler, K. E.; Holland, C.; Goubet, F.; Franková, L.; Houston, D. R.; Hudson, A. D.; Meulewaeter, F.; Fry, S. C. Hetero-trans-β-glucanase, an enzyme unique to Equisetum plants, functionalizes cellulose. *Plant J.* **2015**, *83* (5), 753−769.
(43) Clode, D. M. Carbohydrate cyclic acetal formation and migration. *Chem. Rev.* **1979**, *79* (6), 491−513.
(44) Kim, H.; Ralph, J.; Akiyama, T. Solution-state 2D NMR of ball-milled plant cell wall gels in DMSO-d6. *BioEnergy Res.* **2008**, *1* (1), 56−66.
(45) Toikka, M.; Sipilä, J.; Teleman, A.; Brunow, G. Lignin−carbohydrate model compounds. Formation of lignin−methyl arabinoside and lignin−methyl galactoside benzyl ethers via quinone methide intermediates. *J. Chem. Soc., Perkin Trans. 1* **1998**, No. 22, 3813−3818.
(46) Eriksson, Ö.; Goring, D.; Lindgren, B. Structural studies on the chemical bonds between lignins and carbohydrates in spruce wood. *Wood Sci. Technol.* **1980**, *14* (4), 267−279.
(47) Li, K.; Helm, R. F. Synthesis and rearrangement reactions of ester-linked lignin-carbohydrate model compounds. *J. Agric. Food Chem.* **1995**, *43* (8), 2098−2103.
(48) Balakshin, M. Y.; Capanema, E. A.; Chang, H.-m. MWL fraction with a high concentration of lignin-carbohydrate linkages: Isolation and 2D NMR spectroscopic analysis. *Holzforschung* **2007**, *61* (1), 1−7.
(49) Del Río, J. C.; Marques, G.; Rencoret, J.; Martínez, Á. T.; Gutiérrez, A. Occurrence of naturally acetylated lignin units. *J. Agric. Food Chem.* **2007**, *55* (14), 5461−5468.
(50) Lahtinen, M. H.; Valoppi, F.; Juntti, V.; Heikkinen, S.; Kilpeläinen, P. O.; Maina, N. H.; Mikkonen, K. S. Lignin-rich PHWE hemicellulose extracts responsible for extended emulsion stabilization. *Front. Chem.* **2019**, *7* (871), 1−18.