SEPARATION OF GALACTOGLUCOMANNANS, LIGNIN, AND LIGNIN-CARBOHYDRATE COMPLEXES FROM HOT-WATER-EXTRACTED NORWAY SPRUCE BY CROSS-FLOW FILTRATION AND ADSORPTION CHROMATOGRAPHY

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A simple method to simultaneously recover polymeric carbohydrates, mainly galactoglucomannans (GGM), lignin, and lignin-carbohydrate complex (LCC) from hot-water-extracted Norway spruce wood is presented. The isolation method consists of cross-flow filtration, where high and low molecular mass species are removed, followed by fixed-bed adsorption on a hydrophobic polymeric resin (XAD-16) to remove lignins and lignans. In the second step of fixed-bed adsorption, a phenylic reversed-phase analytical chromatography column, where mass transport resistance is minimized and a very high selectivity towards aromatic compounds have been observed, was used to separate LCC from GGM. The isolated LCC fraction contained about 10% aromatics, whereas the upgraded GGM fraction contained about 1.5% aromatics and the lignin fraction contained about 56% aromatics. Polymeric xylan was accumulated in the GGM fraction, while mannose was the dominant sugar found in the LCC fraction. As products, approximately 7% was recovered in the lignin fraction in the first adsorptive step, 5% was recovered as LCC, and 88% as upgraded hemicelluloses.

Keywords: LCC; Lignin carbohydrate complex; GGM; galactoglucomannan; Norway spruce; Biorefinery; Separation; Sorption; Chromatography; Ultrafiltration

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INTRODUCTION

The dominant species of the large Scandinavian forests is Norway spruce (*Picea abies*), and it is presently utilized in traditional applications, such as paper products, sawn timber, and dissolving pulps. However, major changes in consumer patterns and the need for renewable resources to replace petroleum-based material, lead to the need for novel products and processes based on biomass, such as spruce. The development of such products requires different wood components in relatively pure forms, and in the Wallenberg Wood Science Centre (WWSC), methods for the pretreatment and extraction of wood components have been developed (Azhar *et al.* 2011). Therefore, in biorefinery concepts, selective liquid-phase fractionation of different wood components is highly interesting, and filtration and chromatographic techniques are suitable for this.

A number of new material applications of wood polymers have been described and are under development. Examples of uses are hydrogels (Lindblad *et al.* 2001) and gas barrier films for food packaging produced with the hardwood abundant xylan (Grondahl *et al.* 2004) and softwood abundant galactoglucomannans (GGM) (Hartman *et al.* 2006). Besides the three common components of wood (cellulose, hemicellulose, and lignin), wood composition investigations of dissolved wood have also found lignin covalently bound to sugars (Bjorkman 1954; Koshijima *et al.* 1984; Azuma *et al.* 1981; Lawoko *et al.* 2006). Whether these structures exist in native wood or if this is an artifact of processing is still debated. The term lignin-carbohydrate complex (LCC) was introduced by Björkman (1954). These structures have also presented potential technical applications. Uraki *et al.* (2006) investigated the amphiphilic properties of LCCs and suggested their potential use as a polymeric surfactant or as a substance carrier in pharmaceuticals. In addition, Oinonen (2011) reports on a method for synthesizing LCC structures and has measured the oxygen barrier properties of polymerized LCC and found them to be similar to the properties of synthesized hemicellulose. LCC is thought to be very diverse in molecular weight and to have aromatic branching on a polysaccharide backbone. If these structures could be separated into pure fractions in order not only by molecular size, but also by the ratio of aromatic to carbohydrate constituents, more specific material characteristics could be developed.

Mainly three means of separation are employed to achieve separation of a bio polymer feedstock: filtration, precipitation, and/or sorption. Investigations of various means of filtration to obtain hemicelluloses from thermomechanical pulp (TMP) process waters have been performed by Persson *et al.* (2010) and Andersson *et al.* (2007), resulting in a maximum purity of saccharide content of about 80 %. Lawoko *et al.* (2006) report a precipitative scheme for obtaining four fractions of LCC from ball-milled wood that differ by sugar composition. Sorptive means have long since been used to remove aromatic substances from industry waste waters, and there are numerous evaluations of the cost and performance of such sorbent material (Lin and Juang 2009). The most frequently used sorbents in lignin carbohydrate research are the XAD and DAX series of polystyrene-divinylbenzene and polyacrylic resins from Rohm and Haas (Willfor *et al.* 2003a; Schwartz and Lawoko 2010; Pranovich, *et al.* 2005). The popularity of these sorbents is due to their low cost and preference for aromatic material in ligno-cellulosic suspensions. They are mainly used to remove lignin-related material from carbohydrates, and the sorbate is thus disposed along with the inexpensive sorbent. Willfor *et al.* (2003a) performed a preparative scale separation of TMP process water where ultra-filtration was followed by adsorption on a hydrophobic (XAD) resin and then precipitation, resulting in a GGM of 95 % purity. Takahashi *et al.* (1982) pursued the work of Azuma *et al.* (1981), where the authors used hydrophobic interaction chromatography (HIC) to characterize LCCs that had been fractionated by gel permeation. Takahashi *et al.* managed to perform semi-preparative HIC separation of the same fractions and to quantify the contents of sugar and lignin.

In the preliminary study of this work, a number of hydrophobic sorbent materials were tested for lignin removal from pressurized hot-water extract of Norway spruce in a high pressure chromatography (HPLC) setup. A large difference in separation efficiency with regard to retaining UV_{280} absorbing species was detected. The most efficient sorbent

produced three distinct peak regions when eluting with organic solvent, whereas the retentate of the frequently used XAD resin corresponded almost exactly with the last eluted peak area of the more efficient sorbent, thus retaining only part of the aromatic contents. The three fractions were eluted in a gradient of increasing organic modifier, and the ratio of UV_{280} absorbance to quantitative light scattering signal increased very strongly from the first fraction to the last. Together these observations were interpreted as meaning that the XAD resin adsorbs lignin constituents while the less hydrophobic, LCC constituents were adsorbed on the more efficient resin.

The aim of this work was to test a simple small-scale bio-refinery process that combines ultra-filtration and hydrophobic aromatic sorption in two stages for isolating lignin, LCC, and GGM. The fractions obtained were analyzed to detect differences and to examine the presence of LCC constituents. The purity of GGM in permeate obtained was compared with that obtained by other reported means of separation. The presence of lignin-carbohydrate bonds in the LCC fraction was verified by the method described by Lawoko *et al.* (2006). Reflections on the drawbacks and benefits of the process are presented and compared to reported fractionations of thermo-mechanical pulp process water.

EXPERIMENTAL

Materials

Pressurized hot water extract (HWE)

Sawdust of Norwegian spruce wood was extracted in deionized water at 163 ˚C for 60 minutes, which, according to Song *et al.* (2008), should liberate wood constituents, especially in the region of 5 to 10 kDa. The liquor to wood ratio in the autoclaves was set to 10:1 in 1.1 l autoclaves. Prior to extraction, the autoclaves were vacuum-extracted for 10 minutes followed by pressurization with nitrogen at 5 bar for 10 minutes, in order to extract trapped air and impregnate voids in wood with liquor. After the cook, the autoclave material was filtered on a high mesh wire to retain fibrous material, followed by filtration on Munktell filter paper no. 50 to reduce fouling in the following cross-flow filtration process. Nine autoclaves were used, each loaded with 100 g moist sawdust. A total of 119 g of total dry solids (TDS) was measured in the filtered suspension. The wood contained 8 wt% moist, which amounts to an extraction yield of about 14.4 % or 144 mg g^{-1} o.d. wood.

Resins

A polymeric resin, Amberlite XAD-16, with styrene divinylbenzene structure was acquired from Sigma-Aldrich. XAD-16 was packed in a 7.8*300 mm steel column after a wash and swelling procedure according to the manufacturer's specification. The loaded column was thoroughly washed with water and methanol prior to use. The XAD-16 column will be referred to as XAD hereinafter. An analytical column, XBridge Phenyl 5 µm (4.6 × 250 mm) (XB) was acquired from Waters (Milford, MA) along with a number of 20 mm guard columns of the same sorbent. The major parameters of the resins are tabulated in Table 1.

Table 1. Characteristics of Sorbents

	XAD-16	XB phenyl
$d_{particle}$ [µm]	~400	5
d_{pore} [Å]	100	130
Specific area [m^2/g]	800	185
Matrix	Polystyrene-divinylbenzene	BEH*
Ligand density	N/A	3 µmol/m^2

** Bridged ethane in silica matrix*

Solvents

Milli-Q water was produced in the lab using a Millipore 50 filter setup. All other solvents were of analytical grade. Comparing chromatograms with sequential elution with methanol, alkali, and acetonitrile in various orders did not point out any eluent as having insufficient elution properties. However, methanol and acetonitrile are aprotic. In addition, they did not affect the ELS detector, and they are comparably easy to evaporate. Acetonitrile was efficient as an eluent at a lower concentration than methanol, most likely an effect of π electrons, which the adsorbed aromatic solutes may interact with, thus removing or decreasing the selectivity for the stationary phase. These arguments led to the use of acetonitrile as the organic modifier throughout the study.

Methods

Cross-flow filtration

A cross-flow filtration pilot plant was used for fractionation of the hot-water extract. The system consisted of a 30 L tank with a gear-pump and ceramic micro- and ultra-filtration units for Mini Kerasep Module (Novasep). The support of the ceramic membranes was TiO_2 and Al_2O_3 and the membrane layer was TiO_2 and ZrO_3. Each filter had a total filter volume of 0.76 L and a total filter area of 816 cm^2. The membranes had a service pH in the range of 0 to 14, a service pressure of 0 to 0.6 MPa, and temperature stability up to 100 ˚C.

The filtration plant was operated at a transmembrane pressure of 2.2 bar with the 5 kDa filter and 2.6 bar with the 1 kDa filter. In both filtrations, the temperature of the liquid was about 40 ˚C and the cross flow velocity was set to 1.7 m/s. In the cross-flow filtration pilot plant, 11.5 L of hot-water extract was ultra-filtrated with a cut-off of 5 kDa to remove larger particles. The filtration system had 3 L dead volume. The dead volume was washed three times in series with 3 L deionized water. A retentate volume of 2 L and a permeate volume of 17.5 L were obtained. The 17.5 L of 5 kDa permeate was filtered through a 1 kDa membrane.

At a dead volume of 3 L, the 1 kDa retentate was washed with an equal volume of deionized water. A 1 kDa retentate volume of 3 L and a permeate volume of 17.5. L were obtained. The 3 L retentate volume was used for further study. Figure 1 illustrates the filtration process.

Preparative sorption

A Waters 510 high performance liquid chromatography (HPLC) pump was connected directly to a glass container filled with the suspension of cross-flow filtered hot-water extract by plastic tubing. The inlet tube was end-fitted with a membrane filter at the end submerged into the sample solution to prevent any particulate matter from entering the column. During loading, the solution was stirred with a magnetic stirrer to maintain a homogenous solution. The flow rate was set to 3 mL/min for the XAD column and 300 mL of hot-water extract suspension was loaded per cycle. The column was washed with nine column volumes (CV) of deionized water in the reversed flow direction.

During washing, the outlet was connected to a UV detector that had been equilibrated with deionized water. The wash was considered complete when the UV response had been reduced by 90%. Elution was done with 2 CV acetonitril in the reversed flow direction with a flow rate of 0.5 mL/min, and the eluate was stored in a glass container in a refrigerator. Washing was then again performed with nine CV of deionized water in the reversed flow direction. The first time elution was performed, samples of the eluate were extracted from every 2 mL and the UV absorption of the samples was compared. Complete desorption was estimated at less than one CV. The same sequence was used on the XB column, but the flow rates were set to 0.5 mL/min at loading and washing and 0.2 mL/min at elution.

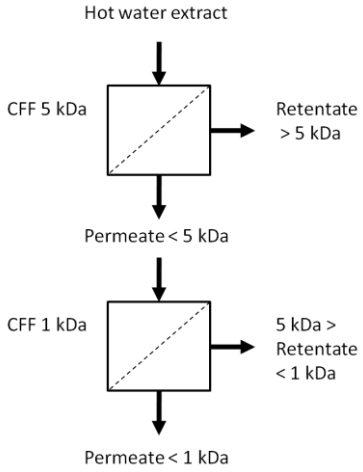

Fig. 1. Cross-flow filtration procedure

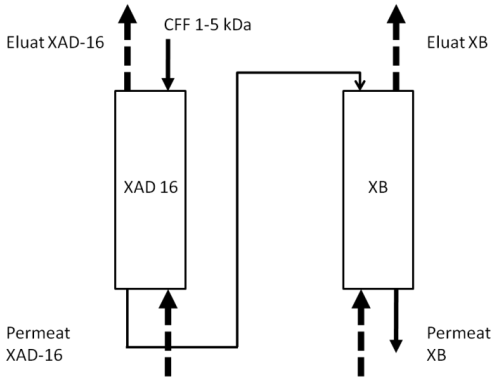

Fig. 2. Preparative sorption procedure.

To assure a minimum of overlap of fractions, two methods were employed to estimate column capacity. During loading, samples of the permeate were collected at void filling, the end of loading, and several times in between and analyzed with a UV spectrophotometer at 280 nm, to detect any increase in aromatic species. To further verify that the sorbent capacity was not exceeded, the permeate was circulated (in the same cycle as above) four times and the permeate UV absorption was measured at 280 nm.

Drying

The fractions obtained and a sample of the original HWE and the permeate from the first sorbent were dried before analysis. Vacuum evaporation was employed with samples in glass flasks submerged in temperate baths kept at 35 ˚C, to accelerate evaporation and prevent the degrading effects of higher temperatures.

Analytical chromatography setup

An analytical system from the manufacturer Waters consisting of a model 600 gradient pump, inline degasser, model 717 autosampler, external column temperature controller, 486 UV detector, and 2424 evaporative light scattering detector (ELSD) was used for the initial testing of sorbents and comparisons of eluates and permeates of the preparative separation.

Enzymatic hydrolysis

A 100 mM phosphate buffer at pH 8 was prepared by mixing 2.95 g sodium dihydrogen phosphate and 53.74 g disodium monohydrogen phosphate in 2 L of water. Stock solutions were prepared by dissolving the dried material in the phosphate buffer to a concentration of 15 g/L. The XAD sample was unable to dissolve completely in the buffer solution, thus lowering the concentration. 30 µL of Novozymes NS-51023 at an enzyme concentration of 30 mg/mL was added to 300 µL vials containing 270 µL of the above stock solutions. The vials were then placed in a heated shaker (~300 rpm) maintaining 47 ºC over 62 hours for the hydrolysis. The activity of the enzyme has previously been tested according to the method described by Lawoko *et al.* 2000, resulting in verified high hydrolysis on glucomannan and no activity on either lignin, pectin, CMC, or xylan (Y. Wang, pers. comm.).

Klason lignin, acid soluble lignin, and sugar analysis

Klason lignin was determined according to the TAPPI test method (1987) T222 OM-83, slightly modified to autoclave at elevated temperature (125 ºC) and pressure (1.4 bar) instead of boiling to complete the hydrolysis of polysaccharides. The acid hydrolysate of Klason lignin was analyzed for neutral monosaccharides using ion chromatography with pulsed amperometric detection (CarboPac PA1 column, Dionex, Sunnyvale, CA, USA). For a more comprehensive description of the analytical procedure, see Wigell *et al.* 2007. The acid-soluble lignin was quantified with spectrophotometry at 205 nm with an absorptivity coefficient of 110 $dm^3\ g^{-1}\ cm^{-1}$.

Size exclusion chromatography (SEC)

The molecular weight distributions of the samples were investigated using a water-based high performance SEC system (Waters 2690) from the Waters Corporation (Milford, MA, USA). The system had an online degasser, an auto sampler, a column oven, and three serially connected columns (Shodex OHpak SB-803 HQ, SB-804 HQ and SB-806 HQ) controlled at 50 °C. The detectors used were refractive index (RI) controlled at 35 °C (Optilab DSP, Whyatt technology corp., Santa Barbara, CA, USA) and a UV-monitor set to record at 280 nm (Shimadzu Corp., Kyoto, Japan). The eluent used was the same 0.1 M pH 8 phosphate buffer used to dissolve the samples and used for enzymatic

hydrolysis. The flow rate was set at 0.4 mL/min. To estimate the molecular weight of the samples, polysaccharides of pullulan were used as the standard. Standards of cellobiose and mannose were used to estimate the molecular mass distribution of hydrolyzed samples.

RESULTS AND DISCUSSION

The main results of the investigation are summarized in Table 2. The complete separation procedure produced three fractions; a retentate on the XAD-16 sorbent, a retentate on the phenylic XB sorbent, and the material that was able to permeate both sorbents without being retained. Throughout the results section these three fractions will be called HWE-XAD, HWE-XB, and HWE-GGM. Hot-water extract, filtered at 1-5 kDa, and the permeate of the XAD sorbent are included in the analysis as points of reference, and are referred to as HWE-CFF and HWE-XAD-P.

Table 2. Summary of Original and Product Streams; Purity and Yield

	Wt% Aromatics	Yield mg g^{-1} O.D wood
HWE-CFF	*5.5*	*49*
HWE-XAD	*55.7*	*1.8*
HWE-XB	*10.2*	*1.4*
HWE-GGM	*1.5*	*23.7**

* Estimated maximum yield from dry matter measurement

A detailed description of composition of the fractions is presented in Table 3, whereas yield is presented in the yield section.

Pressurized Hot-Water Extraction

Hot-water extraction of Norway spruce has been thoroughly investigated by Song *et al.* (2008). These authors pointed out the dependence of the size and shape of the wood to be extracted, where about 70 % more was dissolved from ground wood than from wood chips (160 ˚C, 60 min), and about 100/170 mg g^{-1} from chips/sawdust, respectively. A mixture of sawdust and wood shavings was used in this work, which yield-wise (144 mg g^{-1}) corresponds rather well to the findings of Song *et al.* (2008). The effect of size and shape is attributed to mass transfer resistance, both in terms of impregnation and dissolution. Differences in extraction procedures are also likely to influence extraction yield. The large vessels used in this work require a few minutes to heat to the target temperature, which reduces the effective extraction time. Increased temperature and/or residence time were shown by Song *et al.* (2008) to rapidly reduce the average MMD. In this work, extraction conditions were chosen with reference to the cited work, and it was verified that the selected extraction conditions mainly produced material around 5 kDa, as intended. The sugar composition reported by Song *et al.* (2008) agrees very well with what was measured in this work, with the exception of a lower content of arabinose, which suggests slightly more severe extraction conditions, as Song *et al.* (2008) found arabinose to be the first sugar to be degraded to monomeric form. The lignin content was

reported to be 15 to 20% of TDS by Song *et al.* (2008), which is far more than what was measured in this project (5 to 6%). Once again; some differences are expected due to the instrumental setup of batch extraction, and this work only measured Klason lignin and ASL, whereas Song *et al.* (2008) estimated total lignin content by using UV_{280} measurement. Orsa *et al*. (1997) states that most of the water-soluble aromatic species found in hot-water-extracted spruce are of low molecular weight, and in this work these were removed by filtration at 1 kDa.

Value is added to the isolated hemicelluloses by preserving a high degree of polymerization (DP). Leppänen *et al.* (2010) describe a flow-through PHWE process, which was shown to be an efficient and selective method for extracting hemicelluloses from spruce wood saw meal with a higher average degree of polymerization than the batch-wise PHWE used in this work and that of Song *et al.* (2008). Conserving high DP with the method described by Leppänen *et al.* (2010) was, however, found to significantly reduce yield.

Cross-Flow Filtration

Figures 3 and 4 describe the relative contents of quantifiable species in the various streams of cross-flow filtration. Arabinose and xylose were mainly found in the low molecular mass part of the extract, along with large portions of the lignin. The retentate fractions of 1 and 5 kDa were very similar in composition. The extraction conditions were however adapted to extract material in the region of 1 to 5 kDa. The measured mass-ratio for the retentates was about 1:3 (5 kDa:1 kDa). The acid soluble lignin was at very low amounts and cannot be distinguished in Fig. 3.

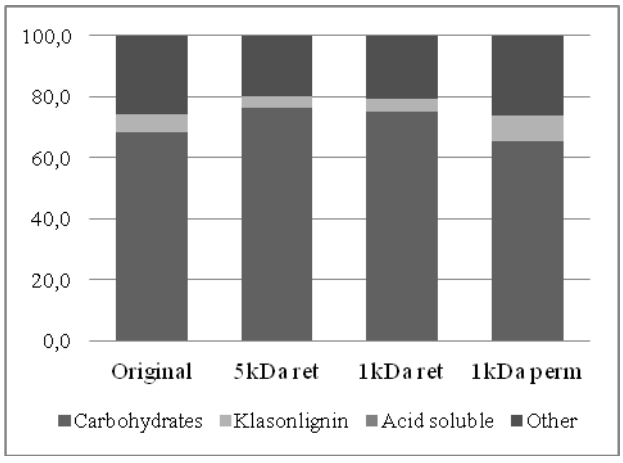

Fig. 3. Composition of cross-flow-filtration samples

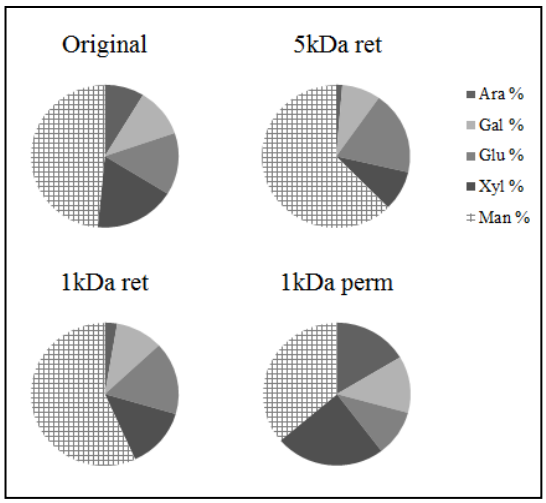

Fig. 4. Sugar composition of cross-flow-filtration fractions

Preparative Sorption

The fractionation observed in the preliminary study did not provide any clues to the capacities of the selected sorbents, which is necessary information in order to maintain purity of fractions and to reduce laboratory workload. Extensive precautions

were made in order to obtain fractions as pure as possible. The loading of HWE was kept far below the estimated capacity (estimated by measuring UV absorption of the permate of the first loading cycle) of the column, and an excess of solvent was used in both the washing and elution sequences. Nevertheless, several tests were employed to further verify the purity of the fractions.

Neither the samples extracted from the permeate during the loading cycle, nor from the circulation tests, exhibited any difference in UV_{280} absorbance. The capacities of the sorbents are thus considered not to have been exceeded. These tests also indicated that the adsorption cycles were performed in the linear part of the assumed adsorption isotherm.

Following elution with acetonitrile, 0.1 M NaOH in 50/50 water MeOH was tested to improve elution, as suggested by Van Loon *et al.* (1993), but no or insignificant amounts of analytes were found in this eluate. Worth noting is that analytical scale retention measurements showed that an increase in the amount of ammoniumsulphate/ ammoniumacetate buffer in the solvent proved beneficial for retention, as suggested by Takahashi (1982). An investigation of the buffer effect was not pursued since this work was intended to simulate industrial processing conditions.

Yield

Exact measurements of mass in fractions was considered to be a subordinate objective partly because the other measurements were a priority due to the small amounts of material obtained and due to the substantial losses required during adsorptive separation in order to maximize the purity of fractions. Thus, the yield must be estimated from other obtained data. The total yield after extraction was measured at 144 mg g^{-1} dry wood after high mesh filtration.

After cross-flow filtration, the remaining TDS amounted to 49 mg g^{-1} where the dead volume of the equipment accounted for a loss of 7.5 mg g^{-1}. The final yield on extract was thus, 34%, which confirms that the HWE conditions chosen could dissolve material in this region, as suggested by Song *et al.* (2008). About 44% of the TDS was lost in the 1 kDa permeate in cross-flow filtration. Excluding losses in handling, the obtained amount of the HWE-DAX fraction was 1.5 g and of the HWE-XB fraction 1.2 g. Consequently, the products from cross-flow filtered HWE could roughly be divided as follows: 7% HWE-DAX, 5% HWE-XB, and 88% HWE-GGM.

Fixed-Bed Degradation

The pressure drops over the columns increased noticeably from the first to the last cycle. Upon dismantling the columns, the frits were noticed to have been severely fouled. Cleansing the frits in heated 0.5 M alkali for 30 minutes removed all visible fouling, and the pressure drop was restored to its original level. It is likely that the fouling occurs during the loading and the elution in pure acetonitrile. In elution, the rapid shift from polar to organic solvent reduces the partition coefficient very fast, which leads to a high concentration of aromatic material in the bulk phase. The high concentration then allows for agglomeration in the bulk phase and the agglomerates are retained at the surface of the frits.

Analysis of Sugar and Lignin Content

The proportion of Man/Glc remains constant at about 4 in all fractions (Table 3), which is in agreement with the Norway spruce glucomannan findings of Willfor *et al.* (2003b). Both galactose and arabinose are strongly reduced in content in the retained fractions. About 12% of the original sugars were xylans. Very small amounts of the xylans were, however, found in the retentates, which lead us to believe that the extracted xylan and arabinogalactan are not bound to lignin. However, previous analyses of lignin carbohydrate complexes in spruce have shown that lignin is bound to both xylan and glucomannan (Lawoko, *et al.* 2005). Thus, it is our belief that a major part of the xylan-lignin complex in spruce wood cannot be extracted by hot water at the conditions applied in this work. This is not strange, as the impediment to the quantitative extraction of hemicelluloses with hot water has partly been attributed to part of them being covalently bonded to a macromolecular hydrophobic lignin in wood (Tunc *et al.* 2010; Chen *et al.* 2010).

Table 3. Detailed Summary of Organic Contents in Original and Product Streams

	Arabinose	Galactose	Glucose	Xylose	Manose	Klason wt%	ASL wt%
HWE-CFF	*3,01*	*10,42*	*15,01*	*11,74*	*59,82*	*4,17*	*1,42*
HWE-XAD	*1,04*	*7,32*	*16,31*	*2,41*	*72,92*	*52,78*	*2,94*
HWE-XAD-P	*3,17*	*10,86*	*14,72*	*12,66*	*58,59*	*1,02*	*0,88*
HWE-XB	*0,20*	*4,55*	*18,46*	*0,55*	*76,24*	*8,48*	*1,71*
HWE-GGM	*3,42*	*11,61*	*14,30*	*13,99*	*56,68*	*0,85*	*0,69*

Sugar composition in % of total sugar. Klason lignin and acid soluble lignin in wt% of TDS.

The sugar composition remained fairly constant in a comparison of the permeates through adsorptive separation; HWE-CFF to HWE-DAX-P, and HWE-GGM. Data consistently showed that the sugars enriched in the retained fractions were reduced in the permeate fraction and vice versa. In both retentates, mannose and glucose were enriched, whereas arabinose, xylose, and galactose were enriched in the permeates. 75.5% of the Klason lignin and 38% of the ASL entering the first sorbent were retained in the HWE-XAD fraction. The XB sorbent reduced the Klason lignin content by 16.6% and the remainder of the ASL by 21.6%. The total reduction of Klason lignin was 79.6% , and the total reduction of ASL was 51.4%.

Following the assumption of Willfor *et al.* (2003a) that all mannose was present in galactoglucomannan with a ratio of mannose:glucose:galactose of 4:1:0.5, the sugar part of the HWE-GGM fraction consisted of about 79 % GGM, and the remainder was xylan and arabinogalactan in the ratio of 2:1. The calculated degree of detection, *i.e.* detectable sugars and lignin, was 80 to 85 % in all fractions. The low degree of detection is likely due to a combination of various measurement errors, mostly attributed to undetected inorganic material in the samples.

SEC Analysis

The elution time of the untreated samples was compared to that of pullulan standards of 5 and 10 kDa. Samples of analytical grade cellobiose and mannose were also included to obtain an approximate retention range of the smallest components expected

after enzymatic hydrolysis. The three obtained fractions, and the original HWE-CFF, all showed an average molecular mass distribution (MMD) between 10 kDa and dimer, with the HWE-XB fraction shifting towards a larger average mass (Fig. 5). Small amounts of the injected samples exhibited extreme retention, which must come from interactions with the column material (not shown). The elution times, consequently, cannot be considered as absolute, instead the relative difference between fractions and between the enzyme-hydrolyzed and untreated samples is compared.

The enzyme hydrolysis proved very effective, leaving an MMD that ranges from only a few degrees of polymerized sugar (DP) to low molecular mass material (< 100 Da). Injecting a sample of untreated enzyme solution on the SEC showed that the low molecular mass material originated mainly from the enzyme solution. The MMDs are similar in all hydrolyzed samples, especially when comparing the UV_{280} detector response. This is interpreted as meaning that the products of hydrolysis are of a similar size and composition. The elution curves are difficult to interpret above an elution time of about 73 minutes (Figs. 5 and 6), where both the residue of enzymatic hydrolysis and low molecular material from the enzyme buffer elutes, and is, therefore, not included in the figures.

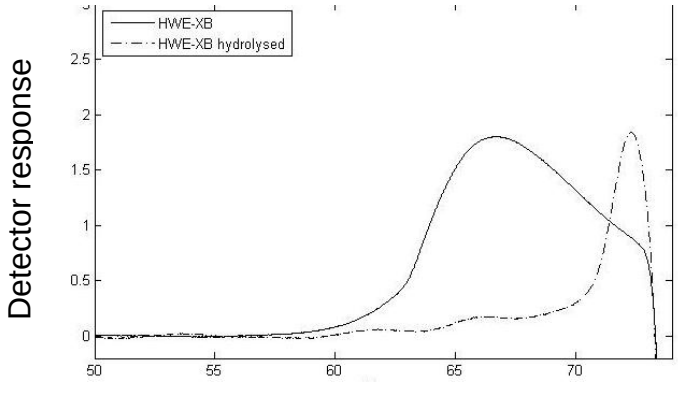

Fig. 5. HP-SEC curve of HWE-XB fractions. RI detector response

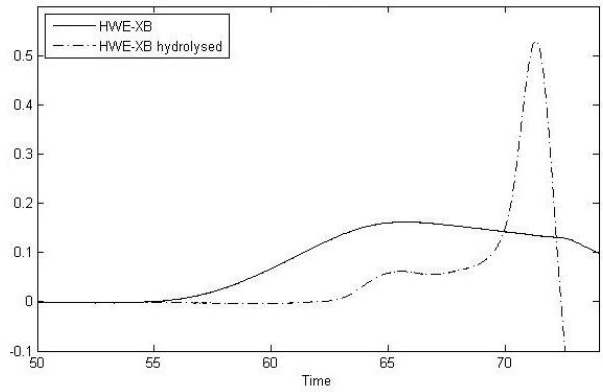

Fig. 6. HP-SEC curve of HWE-XB fraction. UV detector signal at 280 nm

Comparison of an enzymatically hydrolyzed and untreated HWE-XB fraction reveals a significant shift towards a lower hydrodynamic radius of the UV_{280} species after degrading the sugars. Since the enzyme does not show any lignanase activity, the MMD reduction is attributed to cleavage of the glucomannan chain. Reducing the MMD of UV_{280} absorbing species through hydrolysis of the glucomannan chain is a very strong indication of aromatic groups being covalently bound to carbohydrates. The shift in the SEC curves of HWE-XB fraction combined with the fact that the fraction was obtained by hydrophobic adsorption and elution in organic solvent is interpreted as the fraction consisting mainly of LCC structures. In Figs. 5 and 6 the detector signal from the injection of the enzyme, in the same volume and concentration as in the hydrolyzed samples, has been subtracted. Small differences in injection concentration and column degradation occurring over time are known to affect retention time, thus making

subtraction between injections impractical. It cannot be ruled out that part of the peaks in Figs. 5 and 6 between 70 and 75 minutes stems from the enzyme solution. Despite the uncertainties in the SEC curves, a distinct shift towards a lower MMD of the UV_{280} species is observed in the HWE-XB fraction after selective enzymatic hydrolysis of the GGM chain. Drying the HWE-XAD clearly changed its solubility properties. The fraction became only partially soluble in all polar solvents tested as well as in DMSO and acetonitrile. The reason for this change is still unclear but may stem from strong irreversible interactions that occur in the lignin polymer during drying. The other three fractions (HWE-CFF, HWE-XB, HWE- GGM) dissolved completely in the polar solvents tested; phosphate buffers ranging between 6 and 8 in pH, weak alkali, and deionized water. The precipitate part of the HWE-XAD fraction was not further characterized but is thought to correspond to the larger molar mass portion, which consists mainly of aromatics.

The RI detector signals confirm successful hydrolysis of the major part of the HWE-CFF and the HWE-GGM samples. The SEC curves of HWE-CFF and HWE-GGM are very similar, with the HWE-CFF chromatogram being somewhat broader in both the UV and RI. These substance portions are most likely found in the HWE-XAD and HWE-XB fractions after adsorptive fractionation. The low amount of aromatic species in these fractions made it impossible to distinguish the signals from the enzymatically hydrolyzed samples from those of the enzyme and, therefore, the chromatograms are not included.

Comparison to other Means of Bio-refining Norway Spruce

This work could be seen as a proof of concept, with the main objective to show that sequential fixed-bed adsorption can be used to fractionate polymeric wood constituents according to aromaticity. To estimate the performance of separation, the used process must be compared with other processes with the same or similar objectives and similar sources of wood constituents.

About 2.5% of the wood material subjected to thermomechanical pulping is dissolved in the process water. GGM, which represents 15 to 20% of the wood in softwood, constitutes the majority of the material dissolved in water (Sundholm 1999). The amount dissolved varies with processing conditions. According to Thornton *et al.* (1994); about 10% of the GGM in softwood is dissolved. The high solubility of GGM is due to its high degree of acetylation in combination with its relatively low-molar-mass (Hartman *et al.* 2006), which is also the case for hot-water-extracted GGM. Willfor (2003) describes a process where 5 kg acetyl-galactoglucomannan is recovered per ton of thermomechanical pulp. A schematic flow sheet illustrating the process for the recovery of non-fibrous wood material components released from thermomechanical pulps is presented in Willfor *et al.* (2003a). The procedure, after dissolving the TMP, includes the removal of colloidal substances by adding cationic coagulant, and the removal of lignin and lignans using an XAD resin followed by ultra-filtration at 20 kDa to remove low molecular weight material. The purity of the acetyl-GGM was 95%. The average M_w was about 29 kDa and the degree of acetylation was 0.29. Willfor *et al.* (2008) report that their group attempted similar fractionation methods to those applied in Willfor *et al.* (2003a) to recover AcGGM from the process water of two Finnish TMP mills. In reference to unpublished results they report an AcGGM molar purity of 70 to 80 %, with

arabinogalactans and pectin as the main impurities. In a patent application from 2002, Eckerman *et al.* takes biorefining of TMP process waters a step further, describing a full process for the extraction of GGM and the recovery of lignins and lignans (Eckerman *et al.* 2002). The isolation of different substances is performed in a number of sequential stages: 1. particulate matter and fibers are removed by pressing or filtration, 2. non-soluble colloidal wood resins are isolated by extraction, filtration, centrifugation or flotation, 3. aromatic compounds are removed by absorbents or chromatography and, 4. finally, salts are removed by dialysis or reverse osmosis leaving a purified fraction of polysaccharides. Eckerman *et al.* (2002) investigated the removal of lignins and lignans through sorption with an XAD resin and found that lignans were easily removed while a large part of the lignin remained. The presence of LCC structures was not investigated. Persson *et al.* (2010) investigated the isolation of GGM through pure sterical means; in a sequence of drum-, micro-, ultra- and nano-filtration. The work of Persson *et al.* (2010) differs from the above, as they applied the separation directly on the process stream of an existing TMP plant. Process water was extracted at the disc filters downstream of the refiners. The plant in question employs closed-loop water circulation and as a consequence, the TDS of the water contained more than 60% characterized either as suspended matter, ash or others. The product stream consisted of 59% hemicellulose, where most was characterized as GGM, and the rest was characterized as 13% lignin, 7% ash, and 21% un-characterized matter remained as impurities. As in the work of Orsa *et al.* (1997), Persson *et al.* (2010) found that lignin accumulated in the fraction of the lowest molecular weight.

Attempting to characterize the aromatic material, Pranovich *et al.* (2005) dissolved TMP through heat treatment combined with mechanical treatment and used XAD resin to adsorb aromatic species. About 10% of the sorbate was insoluble in methanol after freeze drying and was characterized to contain 11% mannose, glucose, and galactose in a 4.8:1.3:1 relation. The sugars suggest a GGM structure and were suggested to be covalently bound to the aromatic species isolated on the resin. With regard to the application of XAD resins for fractionating lignocellulosics in the solution state (Pranovich *et al.* 2005), to trap aromatics dissolved from TMP, Schwartz and Lawoko (2010) applied it successfully to separate acid soluble lignin and furfural from monosugars resulting from the acid hydrolysis of hemicellulose extracts. In contrast, the present work deals with the separation of a lignocellulosic solution of a polymeric/ oligomeric nature.

Lawoko *et al.* (2006) present a comprehensive precipitative scheme to recover LCCs in fractions that vary in both sugar composition and lignin content. The aim of their work was to characterize lignin-carbohydrate complex structures in wood and to prove the existence of these structures in native wood. Their extensive work presents evidence of lignin being covalently bound to all major polysaccharides in the wood cell wall and that virtually all lignin is bound to polysaccharides. Takahashi *et al.* (1982) used a simpler fractionation method to recover fractions of LCC differing in lignin content. Hydrophobic interaction chromatography (HIC) showed that LCC structures could be eluted exclusively depending on their lignin content. Furthermore, tests of two HIC resins showed the improved retention of LCC species by π- π interaction between aromatic ligands and lignin. The attained fractions were of a sufficient quantity to determine sugar

content. Both the work of Lawoko *et al.* (2006) and of Takahashi *et al.* (1982) showed that LCCs in wood vary in composition and can be fractionated accordingly. The methods used were developed for analytical purposes and were, in that sense, applied successfully, but for industrial separation purposes there is a need for simpler methods that are allowed to compromise fraction purity for economic purposes.

The reported methods for recovering non-fibrous wood constituents have shown limitations in both purity of products and economic feasibility in large-scale separation. The recovery of LCC from wood is a relatively new concept. Sequential fixed-bed adsorption is promising both in terms of process implementation and economy. Among the benefits of the investigated separation procedure are the simplicity of the unit operation and the fact that the method allows for simultaneous isolation of LCC and upgrading of GGM, while aromatic impurities are easily removed in a preceding adsorption unit. Moreover; the absence of chemical agents and mild separation conditions minimize chemical modification of the polymers, thus maintaining a high degree of polymerization, which retains value in the recovered species. From an economic perspective, the removal of water from the LCC fraction is very beneficial as drying is known to be the costliest unit operation in the forest industry. In contrast to the above stated benefits of an adsorptive separation process, such a process has a limited separation capacity, which could lead to equipment of unreasonable size. In addition, the sorbent material is degraded which imposes both the need for including a step to regenerate the fixed-bed and an exchange of sorbent material as lifetime expires.

CONCLUSIONS

This study has demonstrated that a bio-refinery process that combines the steric separation of filtration with the selective separation method of adsorption chromatography can be used to fractionate polymeric lignin, LCCs, and GGM from hot-water-extracted Norway spruce.

For the LCC fraction, the presence of lignin covalently bound to carbohydrates, along with the relative amount of lignin, was considered sufficient characterization, while the aromatic residue in the GGM fraction was determined to be about 1%.

In comparison to other means of purifying LCC and GGM, the method investigated in this study is suggested if more than one of the fractions is regarded as product and when avoiding chemical modification is essential.

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