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Extraction of lignin with high β-O-4 content by mild ethanol extraction and its effect on the depolymerization yield.
--Manuscript Draft--

1 **TITLE:**
2 Extraction of Lignin with High β-O-4 Content by Mild Ethanol Extraction and its Effect on the
3 Depolymerization Yield
4
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25 **KEYWORDS:**
26 Lignocellulose, fractionation, lignin, 2D-NMR, depolymerization, acetals, phenols
27

28 **SHORT ABSTRACT:**
29 Here, we present a protocol to perform ethanol extraction of lignin from several biomass sources.
30 The effect of the extraction conditions on the lignin yield and β-O-4 content are presented.
31 Selective depolymerization is performed on the obtained lignins to obtain high aromatic
32 monomer products.
33

34 **LONG ABSTRACT:**
35 Lignin valorization strategies are a key factor for achieving more economically competitive
36 biorefineries based on lignocellulosic biomass. Most of the emerging elegant procedures to
37 obtain specific aromatic products rely on the lignin substrate having a high content of the readily
38 cleavable β-O-4 linkage as present in the native lignin structure. This provides a miss-match with
39 typical technical lignins that are highly degraded and therefore are low in β-O-4 linkages.
40 Therefore, the extraction yields, and the quality of the obtained lignin are of utmost importance
41 to access new lignin valorization pathways. In this manuscript, a simple protocol is presented to
42 obtain lignins with high β-O-4 content by relatively mild ethanol extraction that can be applied
43 to different lignocellulose sources. Furthermore, analysis procedures to determine the quality of
44 the lignins are presented together with a depolymerization protocol that yields specific phenolic

45 2-arylmethyl-1,3-dioxolanes, which can be used to evaluate the obtained lignins. The presented
46 results demonstrate the link between lignin quality and potential for the lignins to be
47 depolymerized into specific monomeric aromatic chemicals. Overall, the extraction and
48 depolymerization demonstrates a trade-off between the lignin extraction yield and the retention
49 of the native aryl-ether structure and thus the potential of the lignin to be used as the substrate
50 for the production of chemicals for higher-value applications.
51
52 **INTRODUCTION:**
53 For the chemical industry to become sustainable, renewable feedstocks such as lignocellulosic
54 biomass should be used as alternative to current dominant fossil ones[1]. However, to make the
55 use of such feedstocks economically viable, high-value applications should be sought for all its
56 contents. Lignocellulosic biomass can contain about 30 wt% of the lignin that is an aromatic
57 biopolymer for which currently only a few applications have been developed beyond its use as
58 low-value fuel[2]. Therefore, the methodology towards potentially increased-value aromatic
59 components is of major interest to ensure the success of future biorefineries.
60
61 Recent research has focused heavily on the development of novel methodologies for the
62 selective cleavage of the most abundant β-O-4 linkage (**Figure 1a**) in lignin to obtain specific
63 aromatic, typically phenolic, monomers[3-6]. For example, the application of acids between 80 °C
64 to 180 °C is very effective in cleaving the β-O-4 linkage forming aldehyde and ketone fragments[7,8].
65 Our groups and others have recently demonstrated that acidolysis combined with the
66 methodologies to stabilize and trap reactive fragments is extremely potent to obtain phenolic
67 monomers with specific chemical motifs[9-12]. Of these, in particular acetal trapping of reactive
68 aldehydes with alcohols to obtain phenolic 2-arylmethyl-1,3-dioxolanes (acetals) proved
69 powerful due to its relative simple application and the retention of the highly functionalized
70 nature of the lignin monomers (**Figure 1b**)[13,14]. These acetals are obtained from the
71 depolymerization in a ratio that relates to the distribution of the H, G and S monomers present
72 in the parent lignin feedstock.
73
74 Acid catalyzed depolymerization, like many of the most elegant methodologies developed, are
75 relatively mild and do not cleave stronger C-C bonds that occur in lignin[15]. However, C-C bonds
76 become especially abundant when harsh lignocellulose fractionation conditions are applied due
77 to the condensation of reactive fragments released from the cleavage of weaker C-O bonds[16,17].
78 The loss of β-O-4 content from the biomass processing method is clearly demonstrated by the
79 analysis of a range of technical lignins, which were shown to only retain up to 6 β-O-4 linkages
80 per 100 aromatic units[18], while in lignocellulose these numbers range from 45 to 90 linkages per
81 100 aromatic units depending on the source[16]. Turning to milder extraction conditions, lignins
82 can be obtained with the linkage distributions that better reflect the natural lignin. Nevertheless,
83 this demands a trade-off between the extraction efficiency and the quality of the obtained lignin
84 material[17]. This also has precedence in organosolv extraction of lignin, which is a popular method
85 to fractionate lignin. Many variations of this process exist, with the methods employing different
86 temperatures, acid content, extraction times and solvents. Here, the extraction severity has a
87 direct impact on the obtained lignin structure and thus its suitability for further valorization[19-21].
88 For example, organosolv lignin produced by the ethanol based Alcell process, operated for 5

89 years at demonstration scale, had relatively low amount of β-O-4 linkages left as it was operated
90 at relatively high temperature to ensure efficient delignification in order to obtain high quality
91 carbohydrates for bioethanol production. Nevertheless, biobased solvents with little
92 environmental impact like ethanol are preferred and thus the extraction methods that result in
93 lignins of higher value are of interest. Alcoholic solvents are of particular interest as in addition
94 to being the extraction medium they also incorporate into the lignin structure, for example, β'-
95 O-4 (**Figure 1a**)[22], which partly "protects" the structure from undesired cleavage. A suitable
96 method would potentially be first to obtain lignin with a high β-O-4 content and in a sequential
97 step to remove the remainder of the lignin to access high value cellulose.

99 In this manuscript, we describe a straight forward and highly reproducible procedure for the
100 extraction of high β-O-4 lignin by mild ethanol extraction. Depending on the biomass source, this
101 can lead to relatively high extraction efficiency and yield. Procedures for the characterization of
102 the obtained lignin are provided as well as how to "deprotect" the etherified β'-O-4[20].
103 Additionally, an assessment procedure is presented for the potential of these lignins in selective
104 depolymerization procedures that rely on selective cleavage of the β-O-4 linkages. This
105 assessment is performed using the iron(III) triflate catalyzed depolymerization in the presence of
106 ethylene glycol to obtain phenolic 2-arylmethyl-1,3-dioxolanes[23] which demonstrates the link
107 between β-O-4 content in the lignin material and the monomer yields[21]. The results show the
108 balance between high lignin extraction efficiency and the potential of the obtained lignin to be
109 depolymerized to specific aromatic monomers.

111 **PROTOCOL:**

113 **1.   Pretreatment of the Walnut Feedstock before Lignin Extraction**

115 1.1.   Production of cut walnut shells

117 1.1.1.   Feed the walnut shells to a hammer cutter to fracture the shells. Equip the hammer cutter
118 with a 5 mm sieve at the outlet. Collect the fractured walnut shells in a 1 L glass beaker.

120 1.1.2.   Feed the fractured shells to a micro hammer cutter to obtain ground shells. Equip the
121 micro hammer cutter with a 2 mm sieve at the outlet. Collect the ground walnut shells in a 1 L
122 glass beaker.

124 1.2.   Extraction of fatty acids from ground walnut shells

126 1.2.1.   Put 150 g of the cut walnut shells in a 500 mL round-bottom flask. Add 200 mL of toluene
127 and a stirring bar to the round-bottom flask.

129 1.2.2.   Attach a reflux condenser to the round-bottom flask. Heat the mixture at reflux
130 temperature (111 ˚C) with an oil bath for 2 h with vigorous stirring.

132 1.2.3.   Stop the heating after 2 h and let the mixture cool down to room temperature by

133 removing it from the oil bath.

135 1.2.4.   Remove the toluene by filtration (185 mm diameter, 10 µm pore size). Discard the toluene
136 filtrate.

138 1.2.5   Remove the toluene residues by heating the walnut shells overnight in a vacuum oven at
139 80 ˚C and 50 mbar.

141 1.3.   Milling of pre-extracted walnut shells

143 1.3.1.   Place 7 ZrO_2 grinding balls with a 20 mm diameter into a 250 mL grinding bowl made of
144 ZrO_2.

146 1.3.2.   Fill the bowl with 40 g of walnut particles. Add 60 mL of isopropanol to the grinding bowl.

148 1.3.3.   Perform the grinding of the walnut shells with a rotary ball mill. Grind in 4 cycles of 2 min
149 grinding at 27 x g followed by a 4 min pause. Keep the temperature of the bowl below 80 ˚C at
150 any time. Perform no more than 3 batches and let the bowl cool down afterwards.

152 1.3.4.   Collect the finely ground walnut shells into a 500 mL round-bottom flask. Remove the
153 isopropanol by rotary evaporation at 40 ˚C and 125 mbar.

155 1.3.5.   Dry the walnut shells overnight in a vacuum oven at 50 ˚C and 50 mbar.

157 1.3.6.   Sieve the finely ground walnut shells through a 1 mm sieve. Ground the particles that are
158 too large again with the rotary ball mill.

160 **2.   Preparation of the Wood Feedstocks**

162 2.1.   Cutting of the wooden planks

164 2.1.1.   Place the wooden planks under a drill, which is equipped with a flat wood speed drill bit.
165 Collect the wood shavings in a glass beaker.

167 2.1.2.   Place the wooden shavings in a coffee grinder to cut them into smaller pieces.

169 2.2.   Extraction of fatty acids from wood

171 2.2.1.   Perform the extraction of the fatty acids from wood in precisely the same manner as
172 described for walnut shells in Step 1.2.

174 Note: No milling of the wood is performed in the ball mill, as the conditions described in Step 1.3
175 did not result in a reduction of the particle size.

3. Extraction of High β-O-4 Ethanosolv Lignin

3.1. Mild ethanol extraction (Method A)

3.1.1. Put 25 g of the feedstock in a 500 mL round-bottom flask. Add an 80:20 ethanol/water mixture (200 mL), 4 mL of 37% HCl solution (0.24 M) and a magnetic stirring bar to the round-bottom flask.

3.1.2. Attach a reflux condenser to the round-bottom flask. Heat the mixture at reflux temperature with an oil bath for 5 h with vigorous stirring.

3.1.3. Allow the mixture to cool to room temperature by removing it from the oil bath. Filter the mixture (185 mm diameter, 10 μm pore size) and wash the residue with 4 times 25 mL of ethanol.

3.2. Work-up and isolation of lignin

3.2.1. Collect the liquor in a 500 mL round-bottom flask. Concentrate the liquor by rotary evaporation at 40 ˚C and 150 mbar.

3.2.2. Dissolve the obtained solid in 30 mL of acetone. Use an ultrasonic bath if the solid does not dissolve completely.

3.2.3. Precipitate the lignin by adding the mixture to 600 mL of water. If no precipitation occurs, add a small amount of saturated aqueous Na_2SO_4 solution to flocculate the lignin.

3.2.4. Collect the lignin by filtration (185 mm diameter, 10 μm pore size). Wash the lignin with 25 mL of water 4 times. Discard the filtrate if no analysis of the hemicellulose fraction is required. If the filtrate is very turbid, add it to a centrifugation tube and collect the (solid) bottom fraction by centrifugation.

3.2.5. Allow the lignin to air dry overnight. Dry the lignin further in a vacuum oven (overnight at 50 ˚C and 50 mbar).

3.2.6. Determine the yield after the lignin is dried overnight in a vacuum oven.

3.2.7 Determine the lignin extraction efficiency by dividing it with the total lignin content as determined by the Klason method[24].

3.3. Higher temperature ethanol extraction (Method B)

3.3.1. Put 15 g of the feedstock in a 250 mL autoclave. Add an 80:20 ethanol/water mixture (120 mL), 2.4 mL of HCl (0.24 M) and a magnetic stirring bar.

3.3.2. Heat the mixture at 120 ˚C for 5 h with a stirring speed of 5.2 x g. Cool the mixture

221   afterwards in an ice bath.

222

223   3.3.3. Filter the mixture (185 mm diameter, 10 μm pore size) and wash the residue 4 times with
224   15 mL of ethanol.

225

226   3.3.4. Perform further work-up and isolation precisely as described in Step 3.2.

227

228   3.4.   Larger scale higher temperature ethanol extraction of walnut shell (Method C*)

229

230   3.4.1. Put 90 g of finely ground walnut shell in a 1 L high pressure autoclave. Add an 80:20
231   ethanol/water mixture (750 mL) and 6.25 mL of H_2SO_4 (0.12 M).

232

233   3.4.2. Heat the mixture at 120 ˚C for 5 h with a stirring speed of 35.8 x g. Cool the mixture back
234   to room temperature by turning on the reactors cooling system after the 5 h reaction time.

235

236   3.4.3. Filter the mixture (185 mm diameter, 10 μm pore size) and wash the residue 4 times with
237   75 mL of ethanol.

238

239   Note: Using multiple filters saves a lot of time.

240

241   3.4.4. Collect the liquors in 2 equal batches. Perform the work-up and isolation as described as
242   in Step 3.2 with double amount of the solvents for both batches.

243

244   3.5   Control experiments of Step 3.1. (**Method A***) and Step 3.3. (**Method B***) (optional)

245

246   3.5.1. Put the exact some materials as described in Step 3.1.1 but replace the HCl solution with
247   1.67 mL of H_2SO_4 (0.12M). The rest of Step 3.1-3.2 is identical to **Method A**.

248

249   3.5.2. Put the exact some materials as described in Step 3.3.1 but replace the HCl solution with
250   1.0 mL of H_2SO_4 (0.12M). The rest of Step 3.3 is identical to **Method B**.

251

252   **4.   De-etherification of the Lignin (optional)**

253

254   4.1.   Dissolve 1000 mg of lignin in a 24 mL of 1:1 1,4-dioxane/water mixture in a 100 mL round-
255   bottom flask. Add 1 mL of a 37% HCl solution to the mixture.

256

257   4.2.   Add a stirring bar and attach a reflux condenser to the round-bottom flask. Heat the
258   mixture to 100 ˚C with an oil bath for 5 h with vigorous stirring.

259

260   4.3.   Allow the mixture to cool down to room temperature by removing it from the oil bath.
261   Add the mixture to 160 mL of water to precipitate the lignin.

262

263   4.4.   Collect the lignin by filtration (185 mm diameter, 10 μm pore size) and wash the lignin
264   with 25 mL of water 2 times. Allow the lignin to air dry overnight. Dry the lignin further in a

265   vacuum oven (overnight at 50 ˚C and 50 mbar).

266

267   **5.   Analysis of Lignin**

268

269   5.1.   Two-dimensional nuclear magnetic resonance (2D-NMR) analysis

270

271   <mark>5.1.1.   Dissolve 60 mg of dried lignin in 0.7 mL of d_6-acetone.</mark> Add a few drops of D_2O if the lignin
272   does not fully dissolve. Put the mixture in an NMR-tube and take a 2D proton heteronuclear single
273   quantum coherence spectra (HSQC) with an NMR spectrometer with the following parameters:
274   (11, -1), (160, -10), nt = 4, ni = 512[20].

275

276   <mark>5.1.2.   Analyze the obtained HSQC spectra. Adjust the spectra by manual phase corrections on</mark>
277   <mark>both axis until all signals are positive, as this is especially important along the horizontal (f2) axis.</mark>
278   <mark>Perform no baseline corrections. The positions of all the linkages are given in Step 5.1.3 and 5.1.6.</mark>

279

280   5.1.3. Integrate the signals in the aromatic region that correspond to the three different
281   aromatic units (proton numbering as per **Figure 4**). These signals are in the region [(Proton
282   range)(Carbon range)]:
283    $S_{2/6}$:   [(6.48-6.90)(104-109)]
284    $S'_{2/6}$:   [(7.17-7.50)(105-109)]
285    $S_{condensed}$:   [(6.35-6.65)(106-109)]
286   G_2:   [(6.78-7.14)(111.5-116)]
287   G_5:   [(6.48-7.06)(115-120.5)]
288   G_6:   [(6.65-6.96)(120.5-124.5)]
289    $H_{2/6}$:   [(7.05-7.29)(128.5-133)]

290

291   Note: $H_{3/5}$ overlaps with the G_5 signal, and it is assumed that $H_{2/6}$ has the same intensity as $H_{3/5}$.
292   The signal for condensed G overlaps with G_5. If no (or hardly any) G_2 and G_6 signals are present,
293   this indicates that full condensation of G has occurred.

294

295   5.1.4. Calculate the amount of aromatic units with the formula:
296   Total aromatic = $(((S_{2/6} + S'_{2/6})/ 2) + S_{condensed}) + ((G_2 + G_5 + G_6 - H_{2/6}) / 3) + (H_{2/6} / 2)$

297

298   5.1.5. Calculate the percentage of G, H and S units with the following formulas:
299   Ratio S = $(((S_{2/6} + S'_{2/6}) / 2) + S_{condensed})$: total aromatic x 100%
300   Ratio G = $((G_2 + G_5 + G_6 - H_{2/6}) / 3)$: total aromatic x 100%
301   Ratio H = $(H_{2/6} / 2)$: total aromatic x 100%

302

303   5.1.6. Integrate the signals in the aliphatic region signals that correspond to the β-O-4, β-β and
304   β-5 linkages and Hibbert Ketones. These are in the region [(proton range)(carbon range)]:
305   β-O-4$_\alpha$ [(4.76-5.10)(73-77.5)]
306   β'-O-4$_\alpha$ [(4.44-4.84)(81.5-86)]
307   β-O-4$_\beta$ and β'-O-4$_\beta$ [(4.03-4.48)(85-90.5)]
308   β-O-4$_\gamma$ and β'-O-4$_\gamma$ [(3.10-4.00)(58.5-62)]

309   β-5$_\alpha$ [(5.42-5.63)(88-92)]
310   β-5$_\beta$ [(3.36-3.56)(53-54.5)]
311   β-5$_\gamma$ [(3.50-4.00)(62-64.5)]
312   β-β$_\alpha$ [(4.59-4.77)(86.5-89.5)]
313   β-β$_\beta$ [(2.98-3.20)(55.5-59)]
314   β-β$_\gamma$ [(3.75-3.96)(72.5-76)] and [(4.10-4.31)(72.5-76)]
315   HK$_\gamma$ [(4.20-4.30)(66-68)]
316
317   Note: The β-protons of the β-O-4 and β'-O-4 linkages overlap. The structural motifs of these
318   linkages are given in **Figure 1**.
319
320   5.1.7. The total number of linkages per 100 C9 units are all based on the signal of the α proton
321   of the linkages. Calculate the total number of linkages with the following formulas:
322   # β-O-4 linkages = (β-O-4$_\alpha$ + β'-O-4$_\alpha$) / total aromatic x 100
323   # β-5 linkages = β-5$_\alpha$ / total aromatic x 100
324   # β-β linkages = β-β$_\alpha$ / total aromatic x 100
325
326   5.2.   Gel permeation chromatography (GPC) analysis
327
328   5.2.1. Dissolve 10 mg of dried lignin in 1 mL of tetrahydrofuran (THF) (with a drop of toluene as
329   the internal standard). Filter this mixture through a 0.45 μm syringe filter into an autosampler
330   vial with a reduced volume inlet of 0.3 mL. Close the autosampler vial with a cap.
331
332   5.2.2. Inject 20 μL of the sample into a THF GPC. Process the obtained data.
333
334   5.2.3. Correct the obtained signal for the reference signal (toluene). Select the elution volume
335   for the correct range (~200-10000 Da). Calculate mass distribution by the software.
336
337   **6.   Depolymerization of Lignins to Phenolic 2-Arylmethyl-1,3-Dioxolanes (Acetals)**
338
339   6.1.   Place 50 mg of dried lignin in a 20 mL microwave vial as the reaction vessel equipped with
340   a magnetic stirrer. Add 0.85 mL of 1,4-dioxane, 50 μL of ethylene glycol in 1,4-dioxane (0.54
341   mL/mL) and 50 μL of octadecane (internal standard) in 1,4-dioxane (26 mg/mL).
342
343   6.2.   Close the reaction vessel and heat the solution to 140 °C while stirring at 3.8 x g.
344
345   6.3.   When the reaction vessel reaches 140 °C, add 50 μL of Fe(III)OTf$_3$ in 1,4-dioxane (0.1
346   g/mL).
347
348   6.4.   Stir the reactor for 15 min.
349
350   6.5.   Cool the reactor to room temperature and remove the depolymerization liquid as
351   described in Step 7.1.
352

7. Work up and Analysis of Depolymerization Mixtures

7.1. Filter the liquid over Celite (permeability: 2.60-6-50 darcy; particle size: 150 mesh Tyler; sieve retain (140 M US): 2.0-25.0%) and collect in a 2 mL centrifuge tube.

7.2. Concentrate the liquid overnight at 35 °C in a rotational vacuum concentrator.

7.3. Extract the final oil/solid with the following procedure:

7.3.1. Suspended and swell the residue in 0.15 mL of dichloromethane (DCM) by extensive mixing (by vortex), 15 min of sonication and 30 min in automatic wheel.

7.3.2. Centrifuge the samples for up to 10 s using a minispin tabletop centrifuge to ensure that the liquid is at the bottom of the tube (centrifugation speed: 671 x g).

7.3.3. Add 0.75 mL of toluene and mix extensively (by vortex and 10 min sonication).

7.3.4. Centrifuge the samples for up to 10 s using a minispin tabletop centrifuge (centrifugation speed: 671 x g) to ensure that the liquid is at the bottom of the tube.

7.3.5. Separate the light organic liquid from the solid or thick oily residue and filter this liquid over a plug of Celite and collect in a glass vial.

Note: This procedure for suspension/washing is repeated three times, and in the last extraction, 0.5 mL of toluene is used.

7.4. Concentrate the combined organic phases by rotary evaporation (40 °C, 20 mbar).

7.5 Dissolve the oily residue in 1 mL of DCM for gas chromatography flame ionization detector (GC-FID) analysis.

7.6 Perform GC-FID using a GC equipped with an FID detector and use helium as the carrier gas. Standard settings: 1 μL injection, a split ration of 50:1, a helium flow of 0.95 mL/min. Equip the GC apparatus with a HP5 column (30 m x 0.25 mm x 0.25 μm) and run with a temperature profile which starts with a 5 min 60 °C isotherm. Follow-up by a 10 °C/min ramp for 20 min to 260 °C. Hold this temperature for 20 min.

7.7 Integrate the peaks in the spectra manually. The retention times of the peaks are as following: octadecane (21.4 min), H-acetal (19.5 min), G-acetal (20.8 min), S-acetal (23.4 min). Use the obtained values in Step 7.8 to perform the quantification.

7.8 Perform the quantification of G acetal, which is based on a calibration curve with a standard compound isolated using an internal standard (octadecane).

397   Note: Calibration curve: $\frac{grams\ G\ acetal}{grams\ octadecane} = 1{,}94 * \frac{Area\ G\ acetal}{Area\ octadecane} + 0{,}042$ (R²=0,9991)

398

399   Yield G-acetal: $\frac{grams\ G\ acetal}{grams\ lignin} * 100$

400

401   Based on previous results[9,21,23] a response factor was estimated for H and S acetal as 2.19 and
402   1.82, respectively.

403

404   **REPRESENTATIVE RESULTS:**
405   In **Figure 2,** the obtained feedstock after pretreatment are shown (left column). All feedstock was
406   obtained as small chips present apart from beech wood, which was acquired as shavings of
407   suitable particle size for extraction. The lignins obtained after the extraction show a wide range
408   of colors and particle sizes. The lignins obtained from mild treatments (method A and second
409   column **Figure 2**) are typically red/pink in color and acquired as small flakes. When harsher
410   conditions are applied (methods B and C*), the obtained lignins have a brown/brownish yellow
411   color (third and fourth column **Figure 2**). The yield did increase for all the extractions performed
412   under harsher conditions (methods B and C*) compared to milder conditions (Reaction scheme
413   in **Figure 1**, results in **Table 1**). This effect was much more profound for walnut (10.2% increase),
414   beech (8.5% increase) and cedar wood (5.1% increase) compared to pine wood (only 0.5%
415   increase). Based on the lignin content of the biomass before extraction (40.3% for walnut, 28.6%
416   for pine[25], 18.8% for beech[25] and 35.1% for cedar[25]), the lignin extraction efficiency of beech
417   wood is especially high (73.9%), whereas for the other sources lower extraction efficiency was
418   obtained. Methods A* and B*, control experiments with sulfuric acid for methods A and B,
419   showed some clear differences in extraction yield. Mild extraction of walnut shells with sulfuric
420   acid (method A*) gave only a very low yield of 2.6%, which is markedly lower than the extraction
421   with hydrochloric acid (method A) (2.6% and 5.0%). However, with harsher extraction conditions,
422   the extraction with sulfuric acid (method B*) shows a higher yield compared to hydrochloric acid
423   (method B) (19.3% and 15.2%), but it should be noted that sugar traces are present in the product
424   obtained by extraction with sulfuric acid.

425

426   From the NMR analysis of the different lignins (example shown in **Figure 4**), the H/G/S ratio and
427   amount of linkages were determined (**Table 1**). Due to the overlap of the β and γ-protons of the
428   β-O-4 and the β'-O-4 linkage, the amount of linkages is quantified using the α-protons.
429   Additionally, the $G_{5/6}$ and $H_{3/5}$ signals overlap but these can be corrected by adjusting the ratios
430   accordingly using the $H_{2/6}$ signals. Also, a signal corresponding to the γ-protons of the Hibbert
431   Ketones and a signal for oxidized S units, which likely are caused by lignin end-groups, are
432   identified.

433

434   The ratios obtained from NMR show that in general extractions with method B provides lignin
435   with higher S content compared to those with method A in the case that the native material
436   contains S units. Also, the extractions with method B provide lignin with a lower amount of total
437   β-O-4 linkages compared to method A, indicating increased degradation upon increase in
438   temperature. An exception is the walnut lignin obtained from methods methods A and B for
439   which the amount of total β-O-4 linkages was very similar. The number of β-β and β-5 linkages

440   does decrease when harsher conditions are applied, although to a lesser extent. Additionally,
441   NMR revealed that all the lignins obtained after ethanol extraction showed a degree of structural
442   modification of the β-O-4 linkage. These have at least ~50% substitution at the α-OH group,
443   resulting in the α-ethoxylated β'-O-4 linkage. The lignin extraction shows high reproducibility,
444   which was proven by performing the mild extraction of walnut shells (method A) 4 times.
445   Especially, the deviation in the total number of β-O-4 linkages is remarkably small. When the
446   extraction was performed under harsher conditions (methods B and C*), the percentage of α-
447   ethoxylation increased. In the HSQC spectra of beech lignin extracted at harsher condition
448   (method B), a signal for S condensed is visible, which fits perfectly with the significant decrease
449   in the amount of β-O-4 linkages. The walnut extraction performed at large scale (method C*)
450   shows a significant decrease for all linkages and a signal for S condensed is visible in the HSQC
451   spectra. The relatively high yield for the extraction of cedar at mild conditions (method A) is
452   caused by the presence of a substantial amount of fatty acid. Control experiments with sulfuric
453   acid gave good insight in the effect of the acid on the composition of the obtained lignin. With
454   mild extraction conditions (method A*), a very pure lignin was obtained which was similar in
455   composition compared to the other mild extractions (method A). The somewhat lower amount
456   of β-O-4 linkages can be attributed to a less efficient incorporation of ethanol into the lignin
457   framework, resulting in a lower number of β'-O-4 linkages. At harsher extraction conditions
458   (method B*), the differences with the obtained lignin is much more profound compared with the
459   lignin obtained from walnut shells extracted in the presence of hydrochloric acid (method B). The
460   total number of β-O-4 linkages shows a sharp decrease (35 and 74, respectively) and the lignin
461   obtained with sulfuric acid shows a high amount of condensation in the aromatic region (48%),
462   which was determined by the integration of the signals corresponding the $S_{condensed}$ and $G_{condensed}$
463   (Step 5.1.3). This high amount of condensation can be fully attributed to sulfuric acid, as the
464   product obtained from the same extraction with hydrochloric acid showed no condensation in
465   the aromatic region. The composition of the product obtained at harsh larger scale extraction
466   (method C*) shows no big difference with the product obtained at a smaller scale (method B*).
467   The only big difference is the lower amount of condensation in the aromatic region in the large-
468   scale extraction (9%) and subsequently a higher amount of β-O-4 linkages. This difference could
469   be caused by the difference in the heating profile between the different autoclaves.
470
471   The lignins were also analyzed by GPC (**Figure 5**) to provide insight in the molecular weight (**Table**
472   **2**). These reveal that when harsher extraction conditions (method B) are applied, both the weight
473   average molecular weight (M_w) and the polydispersity are increasing for all sources. The number
474   average molecular weight (M_n) between the extraction conditions are comparable for each
475   source. Overall, these results show that harsher extraction conditions have a two-fold effect, and
476   larger fragments are extracted in addition to additional breakdown of such fragments.
477
478   For some applications, the formation of β'-O-4 linkage is undesired, for example, when applying
479   depolymerization methods that rely on the oxidation of the benzylic (α) hydroxyl group[26-28]. The
480   transformation of β'-O-4 linkage of ethanosolv lignin to regular β-O-4 linkages was previously
481   reported[20] and was performed with a lignin batch obtained from walnut shells that is comparable
482   to the lignin obtained from walnut shells reported in this paper (**Figure 6**). This lignin consisted
483   of 30 native β-O-4 linkages and 39 α-ethoxylated β'-O-4 linkages (34 and 38 linkages, respectively

484 for the lignin in this paper). De-etherification converted almost all the α-ethoxylated linkages to
485 the native structure as the obtained lignin consisted of 57 β-O-4 linkages and only 3 α-ethoxylated
486 β'-O-4 linkages, showing a small loss in the total number of β-O-4 units. The mass of the lignin
487 was 72% of the original lignin, which is primarily caused by the loss of the ethyl group.

488

489 To demonstrate the potential of the lignin for the production of aromatic monomers through
490 mild depolymerization, acidolysis reations with $Fe(OTf)_3$ in the presence of ethylene glycol were
491 performed (**Figure 7**). This reaction yields three different phenolic 2-arylmethyl-1,3-dioxolanes
492 (acetals) that relate to the H, G and S units present in the lignin. **Table 3** shows the yield of the S,
493 G and H acetals and the total yields are shown in **Figure 8**. It is visible that the lignin extraction
494 method has a major effect being the yield of acetals. Lower yields are obtained for lignin
495 extracted using harsher conditions (method **B**). This is likely due to a more modified (higher
496 percentage of α-ethoxylation) condensed structure as described in the previous paragraph.

497

498 The importance of the β-O-4 units is reflected by providing correlations to monomer yield in
499 depolymerization such as presented in the protocol (**Figure 9**). A clear trend is visible considering
500 the total β-O-4 content and the non-etherified β-O-4 linkages, where a higher β-O-4 content
501 generally results in higher yield of phenolic 2-arylmethyl-1,3-dioxolanes (acetals) which is in line
502 with previous results[21]. When considering the etherified β'-O-4 linkages, the trend is also clear,
503 showing that the depolymerization yield is not related to the number of β'-O-4 linkages. Under
504 reaction conditions, the etherified β-O-4 linkages can be de-etherified but this additional step
505 results in the loss of material, as described earlier.

506

507 Overall, correcting the monomer depolymerization yield for the lignin extraction yield, the
508 following results can be obtained (**Table 4**). These show that comparing methods **A** & **B**, generally
509 higher amounts of acetal can be obtained by harsher extraction providing higher overall lignin
510 yields followed by a (less selective) depolymerization. Nevertheless, the results for pinewood also
511 show that this is dependent on the biomass source since the increase in extraction severity does
512 not provide a significant yield increase. Retention of the β-O-4 structure is preferred for this wood
513 type to give higher overall phenolic 2-phenylmethyl-1,3-dioxolane (acetals) yields.

514

515 **FIGURE AND TABLE LEGENDS:**
516 **Figure 1. Chemical structures of the obtained products.** (a) Common structural motifs as present
517 in the lignin structure. (b) Acid catalyzed lignin depolymerization combined with acetal trapping
518 to obtain phenolic 2-arylmethyl-1,3-dioxolanes (acetals).

519

520 **Figure 2. Obtained lignin from different feedstocks.** Image of the four different lignocellulose
521 feedstocks after pretreatment (Steps 1 and 2) and the obtained lignins after organosolv
522 extraction at different conditions (method A-step 3.1, method B-step 3.3 and method C*-step
523 3.4).

524

525 **Figure 3. Reaction scheme for ethanosolv extraction**. Overview of the obtained linkages: β-O-4
526 (R' = H), β'-O-4 (R' = Et), β-β and β-5. Conditions: (A) 80 ˚C, 0.24 M HCl (step 3.1), (B) 120 ˚C, 0.24
527 M HCl (step 3.3), (C*) 120 ˚C, 0.12 M H_2SO_4 (step 3.4) and the control experiment A* and B* (step

528   3.5).
529
530   **Figure 4: HSQC analysis of lignin.** Identification of all lignin linkages measured with 2D-HSQC of
531   lignin obtained from walnut shells using mild treatment (step 3.1). The signals for HK_γ and $S'_{2/6}$
532   are magnified to make them visible.
533
534   **Figure 5: Molecular weight of lignin.** GPC graphs of the obtained lignins divided by source (**a** =
535   walnut, **b** = pine wood, **c** = beech wood and **d** = cedar wood). The lines correspond to different
536   samples as given by **Table 2**.
537
538   **Figure 6: De-etherification of lignin.** Reaction scheme of the de-etherification of the obtained
539   ethanosolv lignin from walnut shells (step 4).
540
541   **Figure 7. Lignin depolymerization to acetals.** Reaction scheme for depolymerization of lignin to
542   phenolic 2-arylmethyl-1,3-dioxolanes (acetals). H unit: $R^1 = R^2 = H$; G unit: $R^1 = OMe$, $R^2 = H$; S
543   unit: $R^1 = R^2 = OMe$ (step 6).
544
545   **Figure 8. Acetal yield per source.** Yields of phenolic 2-arylmethyl-1,3-dioxolanes (acetals)
546   obtained from depolymerization of lignin from different sources.
547
548   **Figure 9. Influence of β-O-4 linkages on the acetal yield.** Yields of phenolic 2-arylmethyl-1,3-
549   dioxolanes (acetals) obtained from lignin depolymerization compared to the total β-O-4 (blue),
550   non-etherified β-O-4 (orange) and etherified β-O-4 (gray) content in the lignin feedstock.
551
552   **Table 1. Ethanosolv extraction results**. Obtained yields, aromatic distribution and linkages for
553   the different extractions performed on biomass. *Sulfuric acid is used as acid. [1]Yield of lignin
554   (wt%)/Lignin content in the feedstock as determined by Klason lignin determination.
555   [2]Hemicellulose and S-condensed present in the product. [3]32% of the S-units are condensed.
556
557   **Table 2: Molecular weights of the obtained lignins.**
558
559   **Table 3: Acetal yields of lignin depolymerization.** Yields of phenolic 2-arylmethyl-1,3-dioxolones
560   (acetals) obtained from depolymerization of lignin from different sources. Conditions: 50 mg
561   lignin, 60 wt% ethylene glycol, 10 wt% Fe(OTf)$_3$, solvent: 1,4-dioxane, 140 °C (1 mL total volume),
562   15 minutes (step 7).
563
564   **Table 4: Overall acetal yield corrected with extraction yield.** Yields of phenolic 2-arylmethyl-1.3-
565   dioxolanes (acetals) obtained from depolymerization of lignin from different sources corrected
566   for lignin extraction yield. [1]Calculation: 100*(lignin yield/100)*(total acetal yield/100).
567   Conditions: 50 mg lignin, 60 wt% ethylene glycol, 10 wt% Fe(OTf)$_3$, solvent: 1,4-dioxane, 140 °C,
568   15 min (reaction via step 6 & work-up via step 7).
569
570   **DISCUSSION:**
571   The results from the extractions at different conditions and from different biomass sources reveal

how the optimal conditions for lignin extraction with a relatively high content of β-O-4 linkages can vary depending on the source. For example, walnut extraction at harsher conditions (method B) provides a three-fold increase in the yield with a near retention of the amount of β-O-4 units, while for beech and cedar the yield increases but is accompanied by a significant drop in the amount of β-O-4 units. On the other hand, for pine, the harsher extraction conditions provide very little benefit in yield and also lead to a lignin with a very low amount of β-O-4 units. This means that typically some form of optimization has to be involved to get the correct balance between the lignin yield and quality in the form of retention of the amount of β-O-4 units in the obtained lignin material.

The large increase in the M_w of the lignin material obtained from harsher extraction conditions demonstrates that under these conditions larger fragments can be extracted providing higher yields. However, at these conditions, additional fragmentation occurs, providing additional lower molecular weight material and thus increasing the polydispersity as can be clearly seen in the GPC graphs of walnut (**Figure 5a**) and cedar (**Figure 5d**) in the form of the signal at ~500 Da.

HSQC NMR is an important informative tool to provide comparative data on the quality of different lignins. It should be noted that in this procedure a standard HSQC experiment is performed, this is great for obtaining comparative data but is not necessarily quantitative due to differences in relaxation times. The high amount of linkages displayed for some lignins in **Table 1** are overestimated. Quantitative HSQC experiments provide better results but cost significantly more NMR time, although alternatives exist[29]. In our experience, the numbers in **Table 1** should be divided by a factor of a 1.3 to better reflect the actual amount of β-O-4 units per 100 aromatic units.

As previously mentioned, the reported results point out how finding optimum conditions can vary depending on the source in order to obtain the maximum monomer yields. For instance, when walnut is used as the starting material, the overall total acetals yield increases around two times if harsher conditions (method B) are employed for lignin extraction. However, this is mainly due to the great difference in lignin extraction yield, without affecting the β-O-4 content. Differently, when pine is used milder extraction conditions (method A) are preferable. In fact, lignin extraction results in very similar yields in the two cases but harsher conditions cause a drop in β-O-4 units (especially non-etherified β-O-4 linkages) which can be the reason for such a low monomer yield, as indicated in the previous paragraph. A significant loss of non-etherified β-O-4 linkages can be observed as well in the cases of beech and cedar if conditions (method B) are applied for extraction which possibly leads to a lower monomers yield. However, the overall acetal yield does not differ that much depending on the extraction conditions. In fact, an approximate two-fold increase in lignin extraction yield is observed for both biomass sources switching from methods A to B which compensates for the roughly two-fold decrease in monomer yield.

DISCLOSURES:

The authors report no conflict of interest

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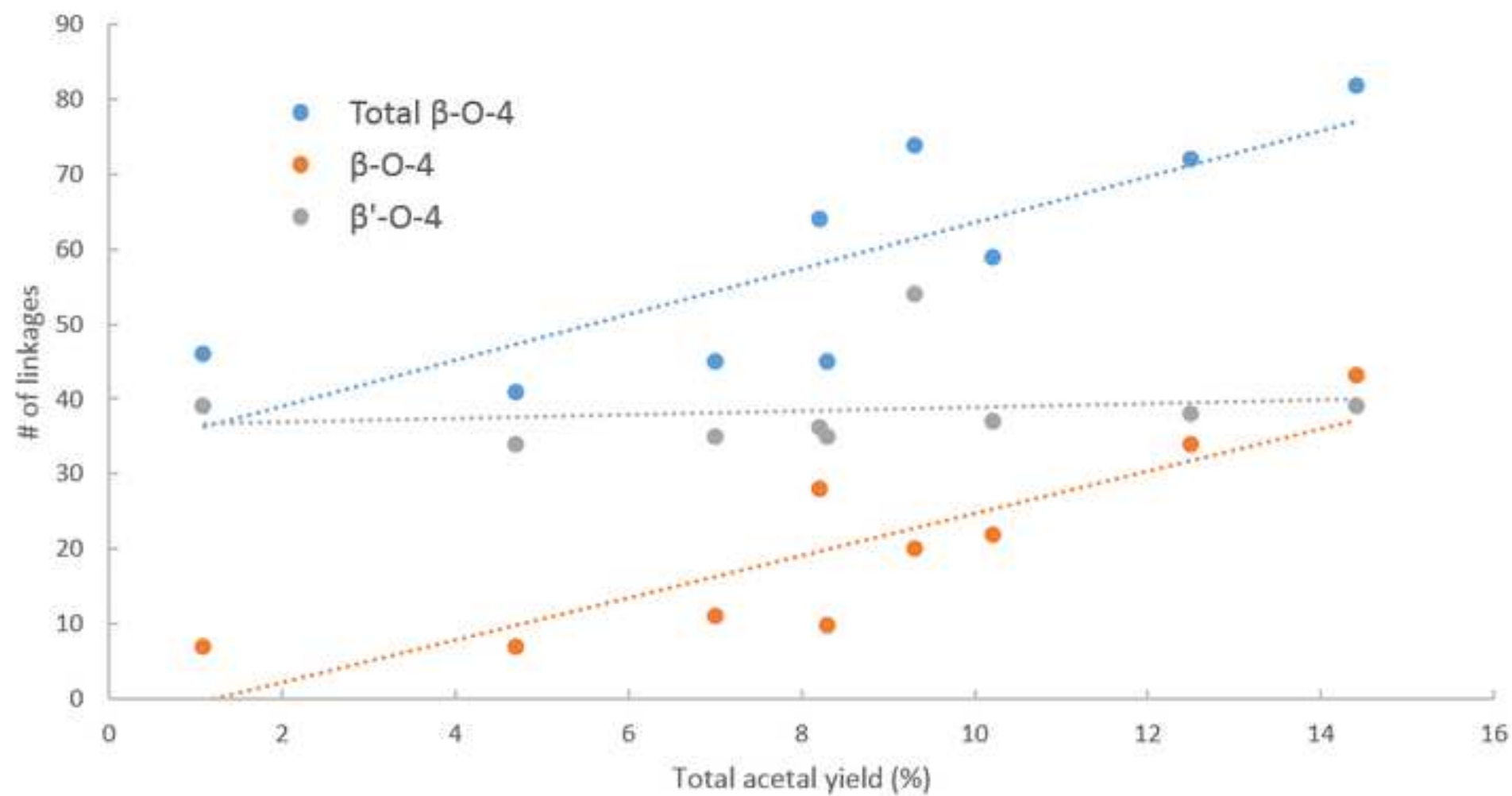
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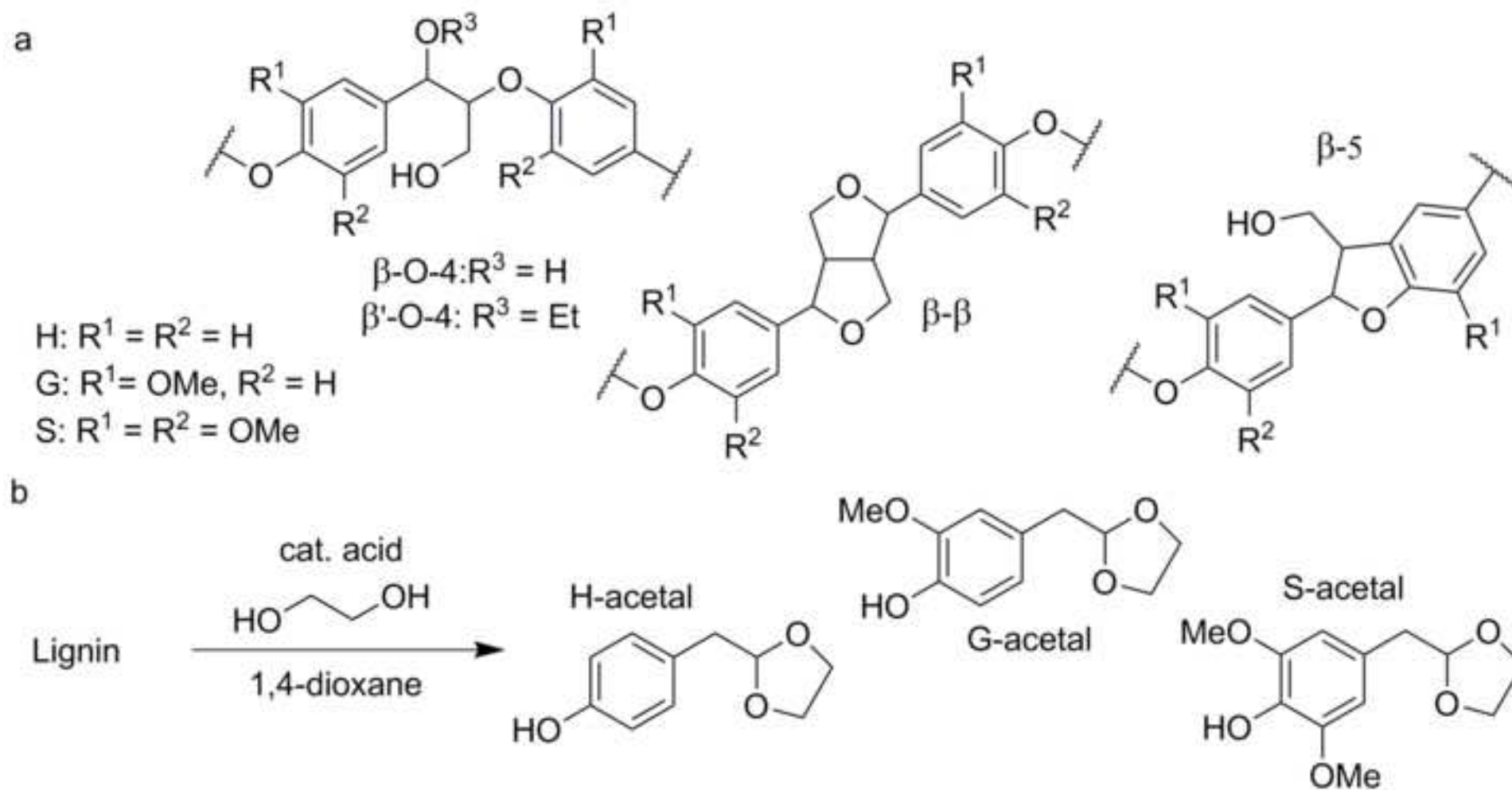
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a

β-O-4: R^3 = H
β'-O-4: R^3 = Et

H: R^1 = R^2 = H
G: R^1 = OMe, R^2 = H
S: R^1 = R^2 = OMe

β-β

β-5

b

Lignin

cat. acid
HO⌒OH
1,4-dioxane

H-acetal

G-acetal

S-acetal

	Feedstock	80 °C (A)	120 °C (B)	120 °C (C*)
Walnut				
Pine				
Beech				
Cedar				

Lignocellulose
4:1 EtOH/H$_2$O
5 hours
A, A*, B, B* or C*

R = H, OMe
R' = H, Et

β-O-4   +   β-β   +   β-5

Lignin $\xrightarrow[\text{HO}\diagdown\diagup\text{OH}]{\text{Fe(OTf)}_3 \quad \text{1,4-dioxane}}$

15 min, 140 °C

R^1, R^2 = H or OCH_3
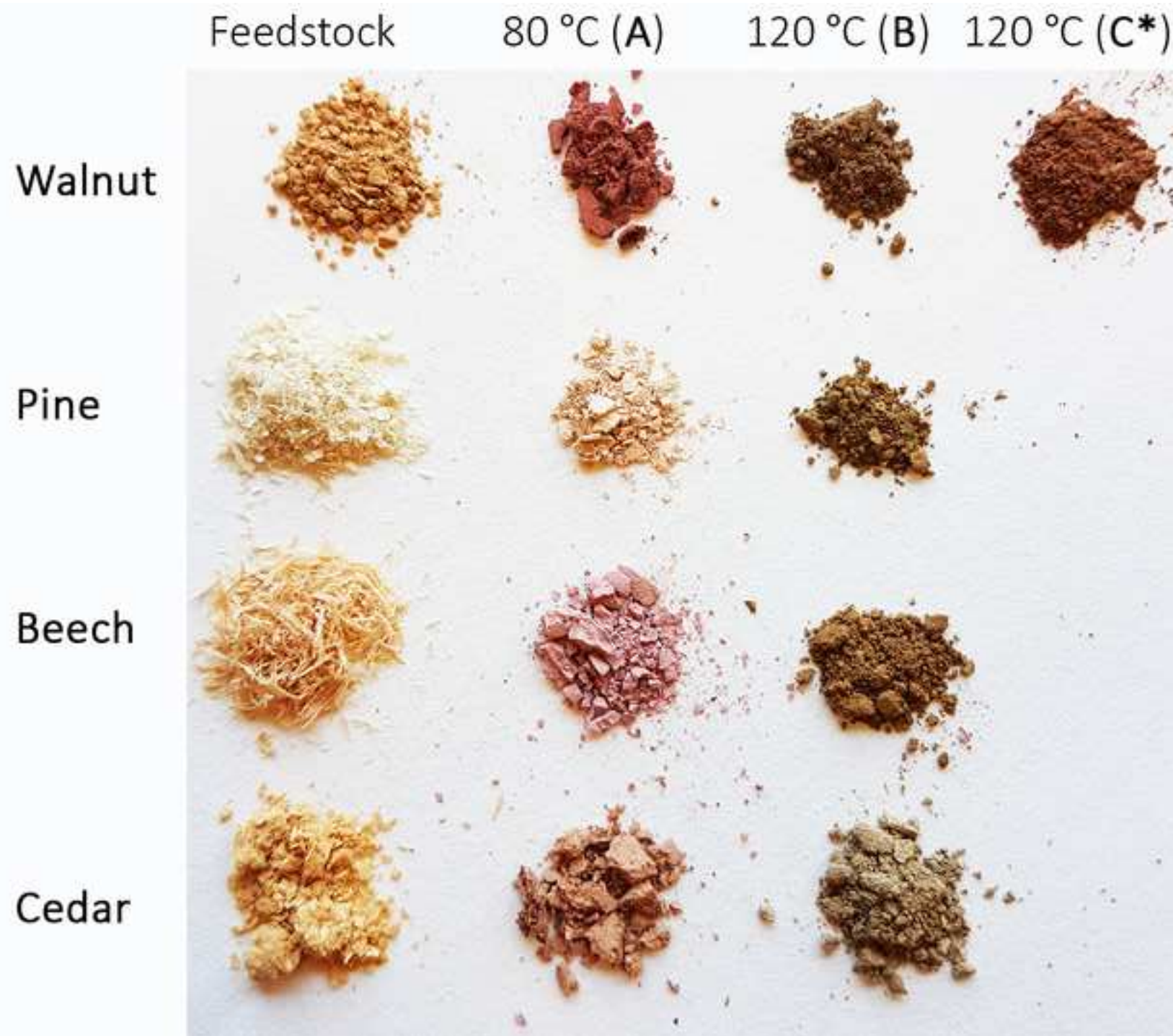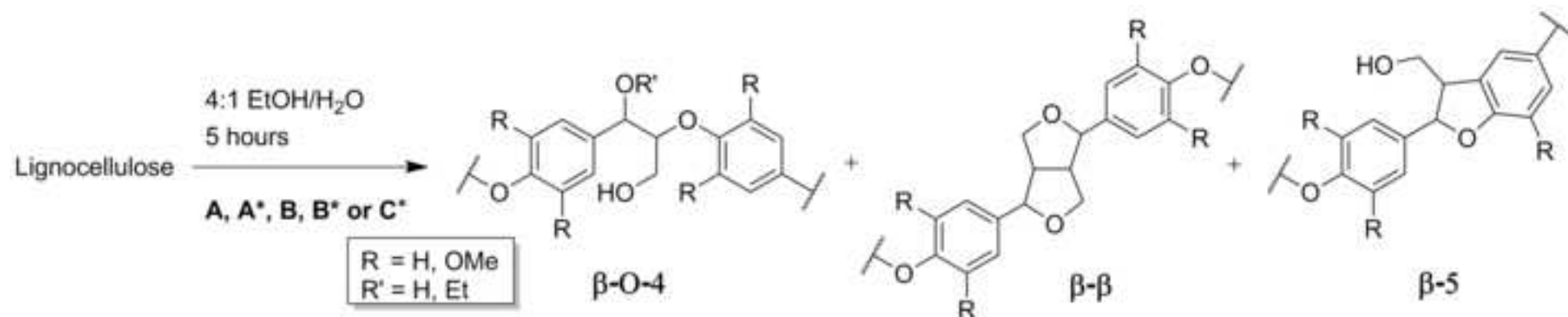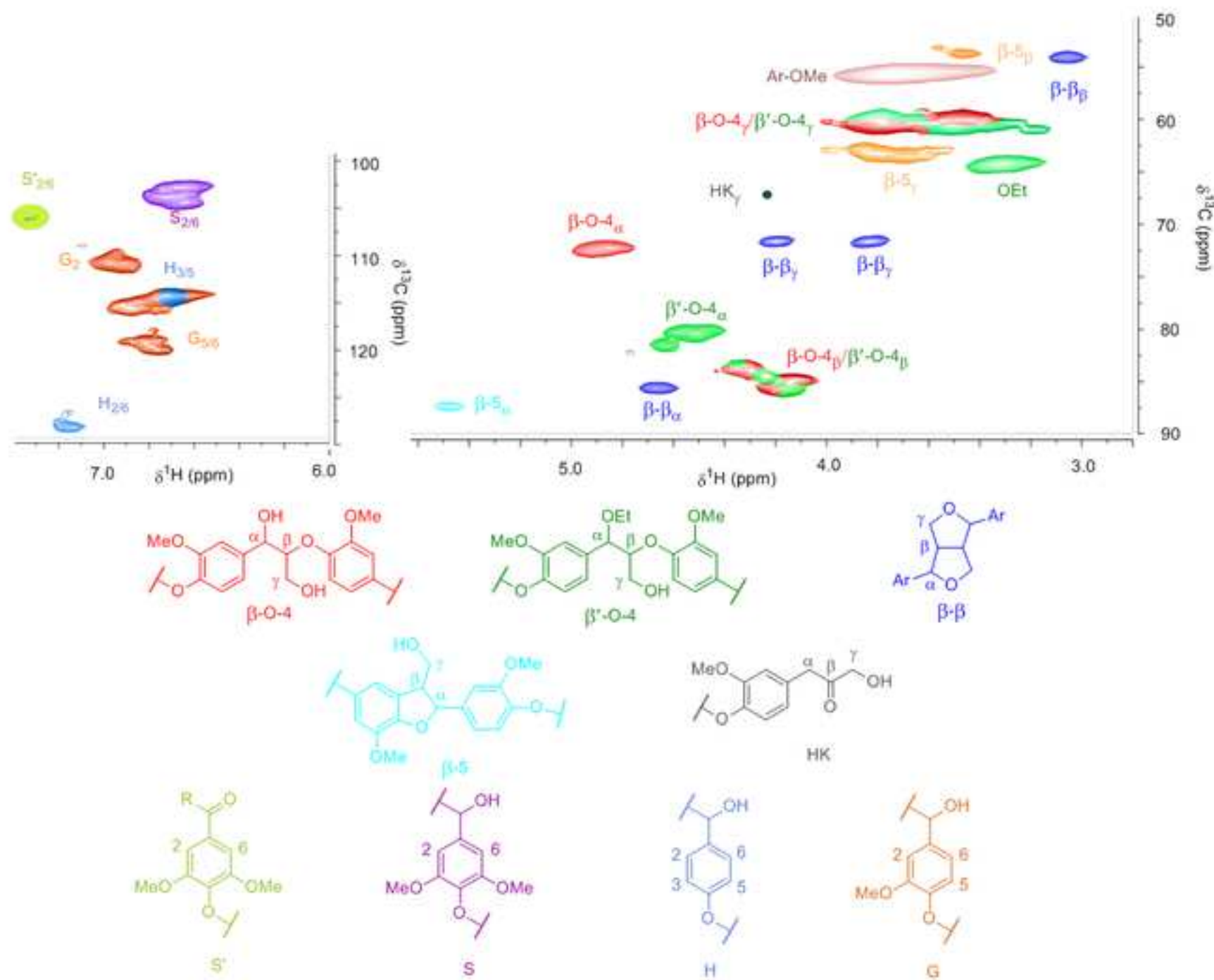
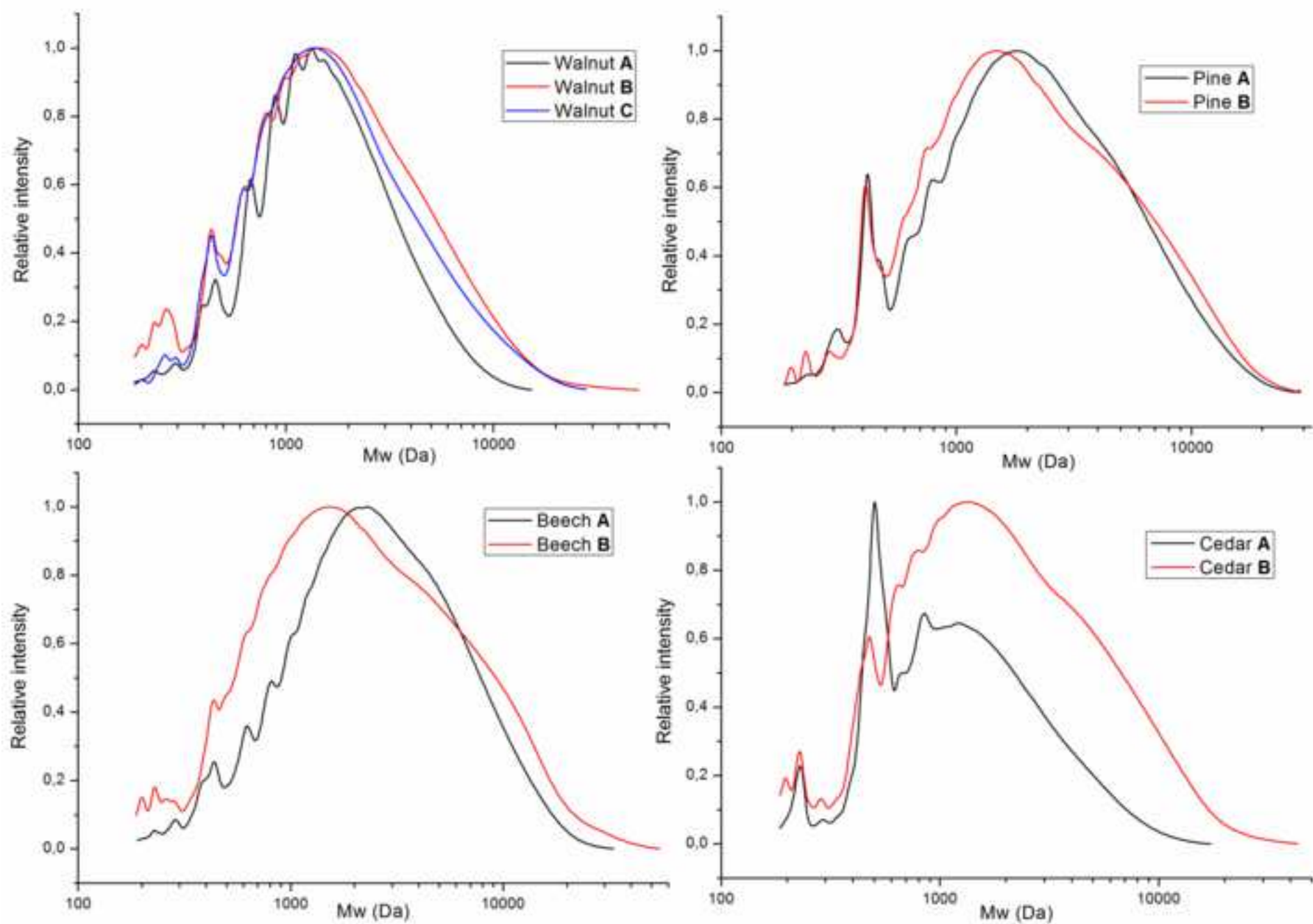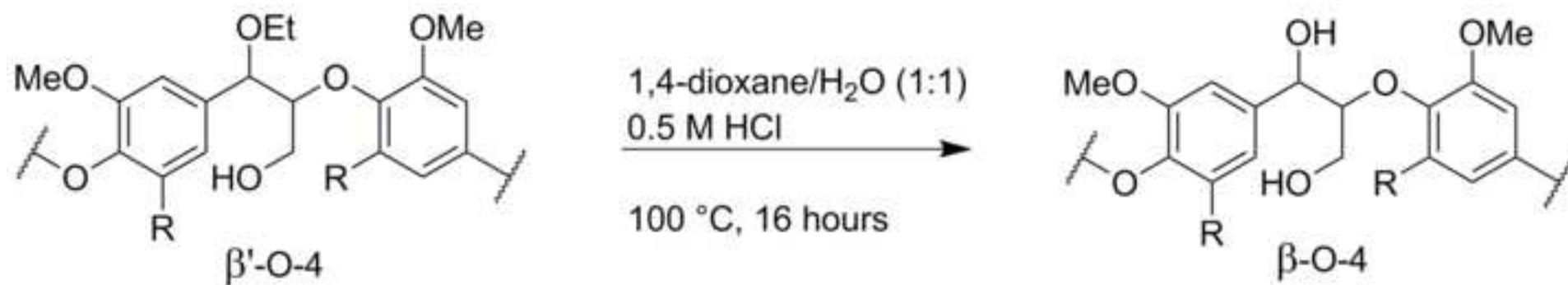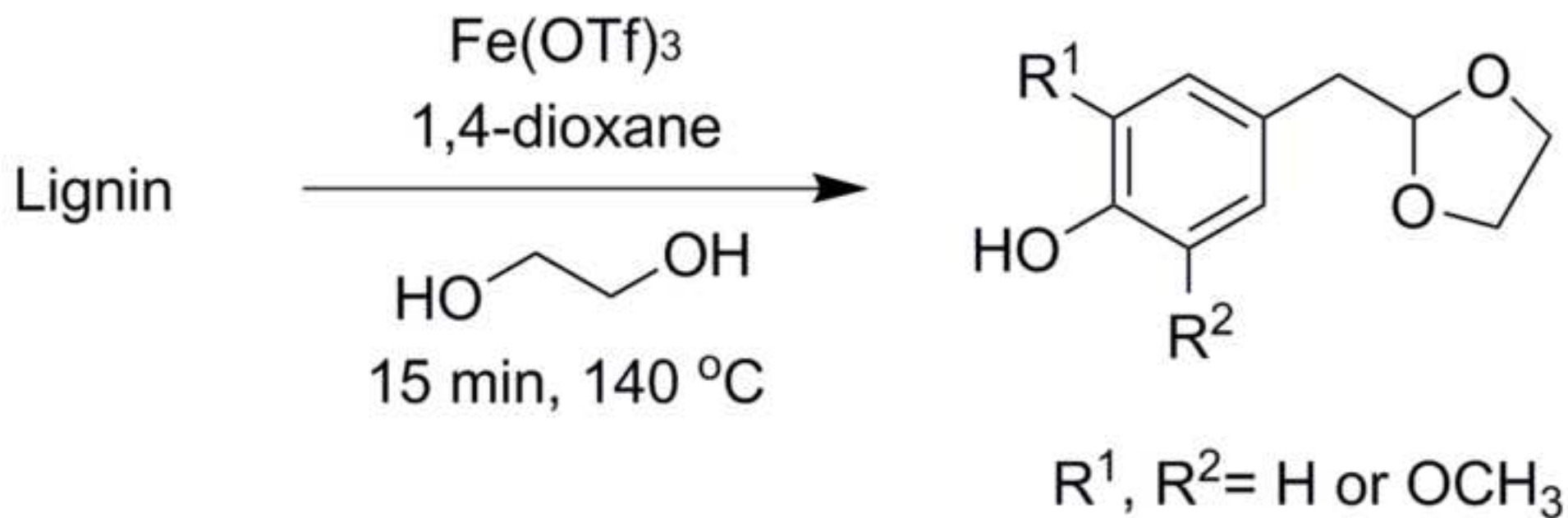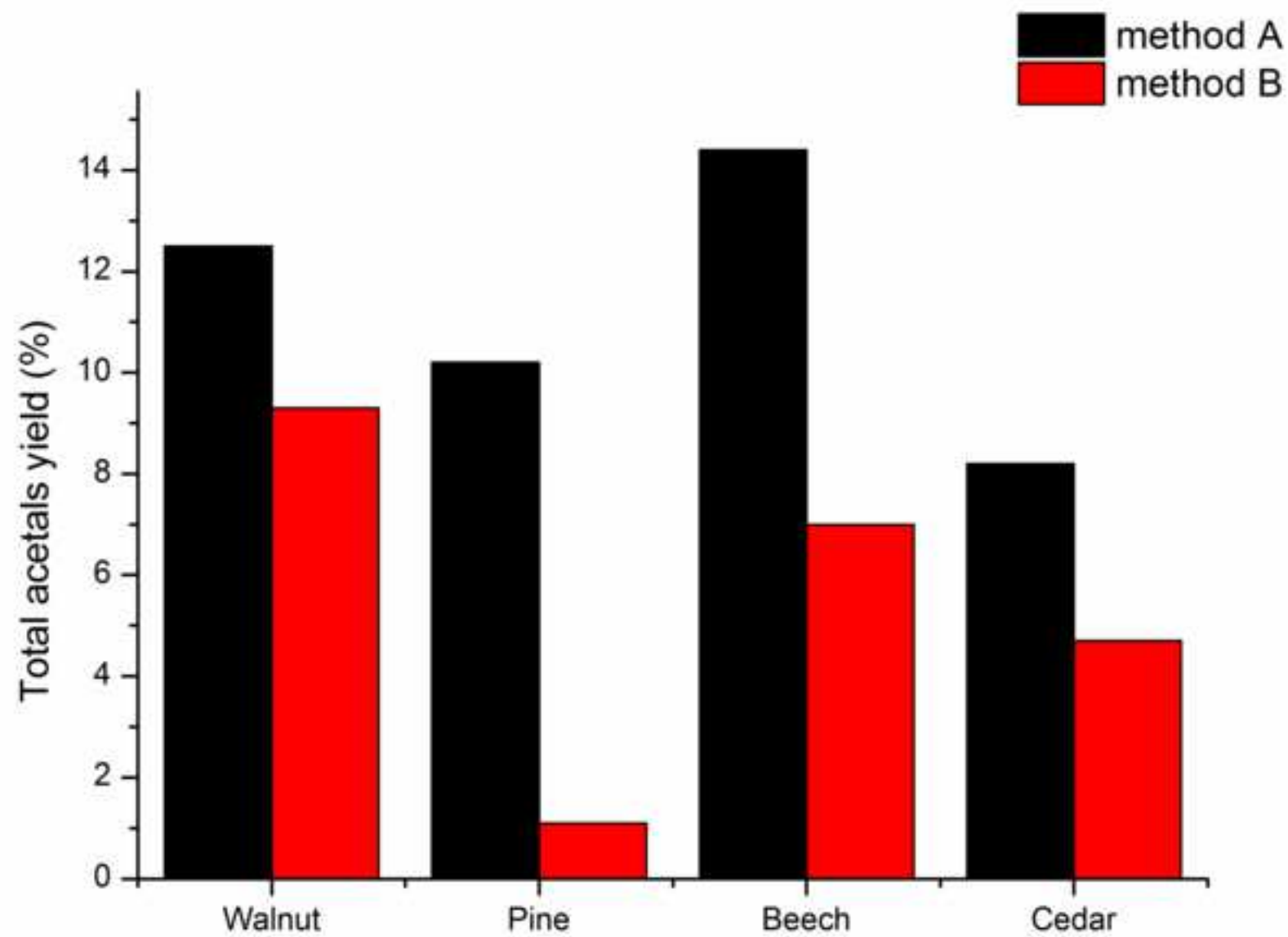| Source	Conditions	Yield (%)	Extraction efficiency (%) [1]	S/G/H ratio	Total β-O-4	β-O-4	β'-O-4
Walnut	A	5.0 ± 0.7	12.4	45/46/9	75 ± 2.5	36 ± 2.6	39 ± 3.1
Walnut	A*	2.6	6.5	47/45/8	53	32	21
Walnut	B	15.2	37.7	59/37/4	74	20	54
Walnut [2]	B*	19.3	47.9	75/25/0	35	5	30
Walnut [2]	C*	16.2	40.2	65/33/2	45	10	35
Pine	A	3.5	12.2	0/>99/<1	59	22	37
Pine	B	4.0	14.0	0/>99/<1	46	7	39
Beech	A	5.4	28.7	63/37/0	82	43	39
Beech [3]	B	13.9	73.9	83/17/0	45	11	35
Cedar	A	6.4	18.2	0/>99/<1	64	28	36
Cedar	B	11.5	32.8	0/>99/<1	41	7	34

β-β	β-5
11 ± 0.7	5 ± 1.5
9	4
9	6
7	3
8	3
0	14
0	8
12	5
9	2
0	6
0	7

Source	Conditions	M_n (g/mol)	M_w (g/mol)	Đ
Walnut	A	1096	1805	1.65
Walnut	B	1174	2934	2.50
Walnut	C*	1248	2930	2.35
Pine	A	1331	3071	2.31
Pine	B	1319	3596	2.73
Beech	A	1645	3743	2.28
Beech	B	1368	4303	3.14
Cedar	A	860	1626	1.89
Cedar	B	1188	3292	2.77

Table

Source	Conditions	Yield (%)	S/G/H ratio	Total β-O-4	S acetal (wt%)	G acetal (wt%)	H acetal (wt%)
Walnut	A	5.0	45/46/9	72	4.5	5.9	2.1
Walnut	B	15.2	59/37/4	74	3.6	4.7	1.0
Walnut	C*	16.2	65/33/2	45	3.8	3.9	0.6
Pine	A	3.5	0/>99/<1	59	0	9.9	0.3
Pine	B	4.0	0/>99/<1	46	0	1.1	0
Beech	A	5.4	63/37/0	82	7.7	6.7	0
Beech	B	13.9	83/17/0	45	3.6	3.4	0
Cedar	A	6.4	0/>99/<1	64	0	8.1	0.1
Cedar	B	11.5	0/>99/<1	41	0	4.7	0

Total acetal yield (wt%)
12.5
9.3
8.3
10.2
1.1
14.4
7.0
8.2
4.7

Source	Conditions	Lignin extraction yield (%)	β-O-4	β'-O-4	Total β-O-4	Total acetal yield (wt%)
Walnut	A	5.0	34	38	72	12.5
Walnut	B	15.2	20	54	74	9.3
Walnut	C*	16.2	10	35	45	8.2
Pine	A	3.5	22	37	59	10.2
Pine	B	4.0	7	39	46	1.1
Beech	A	5.4	43	39	82	14.4
Beech	B	13.9	11	35	45	6.9
Cedar	A	6.4	28	36	64	8.2
Cedar	B	11.5	7	34	41	4.7

Source	Conditions	Lignin extraction yield (%)	β-O-4	β'-O-4	Total β-O-4	Total acetal yield (wt%)

Overall acetal yield corrected for lignin extraction yield (wt%)[1]
0.63
1.41
1.33
0.36
0.04
0.78
0.96
0.52
0.54

Name of Material/ Equipment	Company	Catalog Number
Materials		
Iron (III) triflate	Sigma Aldrich	708801-1G
Octadecane	Sigma Aldrich	0652-100G
Celite	Alfa Aesar	H33152.0B
Silica Gel	SiliCycle	R12030B-1KG
Dichloromethane	Macron Fine Chemicals	6779-25
Walnut shells		
Pine wood		
Cedar wood		
Beech wood		
Ethanol	JT Baker Chemicals	00832000001
Isopropanol	Acros Organics	149320025
Acetone	Macron Fine Chemicals	2440-06
Tetrahydrofuran	Boom B.V.	164240025
Toluene	Macron Fine Chemicals	8608-02
Water		
1,4-Dioxane	Acros Organics	408820010
Hydrochloric acid	Acros Organics	124620026
Sulfuric acid	Boom B.V.	760519081000
Acetone-d6	Acros Organics	325320500
Deuterium oxide	Sigma Aldrich	151882-100G
Filters	Munktell	400303185
Magnetic stirring bars	VWR	442-4525
Syringe filter	Sartorius	17559-Q
Autosampler vial (2 mL)	Brown	151123
Reduced volume inlet (0.3 mL)	Brown	150820
Autosampler caps (11 mm)	Brown	151216
Autosampler vial crimper		
Oil bath		
Syringes (1 mL)	Henke Sass Wolf	4010-200V0
Heating block-4 positions	IKA	

Micro tubes 2 ml	Sarstedt	72691
Crimp seals-20 mm	Brown Chromatography Supplies	151287

Equipment

Rotary Ball Mill	Fritsch	06.2000.00
Hammer mill	Brabender	
Micro Hammer mill	Brabender	
Vacuum oven	Heraeus	
Reflux setup and other glassware	CBN Suppliers B.V.	
Rotary evaporator	IKA	
250 mL high pressure autoclave	Berghof	
1 L high pressure autoclave	Medimex	
Ultrasonic bath	Emerson	
NMR instrument	Bruker	
THF-GPC	Hewlett Packard	
Magnetic stirring plate	SalmenKipp	SK861492220263
Coffee grinder	Profi Cook	PC-KSW1021
Drilling machine	Solid	
GC-FID	Shimadzu	
BUCHI Reveleris PREP purification system	Buchi	
BUCHI C18 column	Buchi	
20 ml microwave vials	???	
Univapo 150 ECH rotational vacuum concentrator	UniEquip	
Eppendorf minispin tabletop centrifuge	Eppendorf	
SB2 rotator	Stuart	
Vortex	Wilten	

Processing Software

WinGPC Unichrom

MestReNova

Comments/Description

purity: 90%
purity: 99%

P60 40-63 µm

Ethanol absolute
99.5+% extra pure

stabilized with BHT

Demi water from the internal supply
99+% extra pure
37% solution in water
95-97%
99.8 atom% D
99.9 atom% D
185 mm diameter, 10 µm pore size

0.45 µm filter

with Silicone/PTFE septa

Laboratory Planetary Mono Mill PULVERISETTE 6

Heraeus Vacutherm
Reflux condensor, Roundbottom flask, Beaker glass and funnels

type Branson 3210
Ascend 600
1100 series
type x-1250

type TB 13 S

150 mm × 21.2 mm × 10 µm

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Title of Article: the effect on the depolymerization yield.
mild ethanol extraction of lignin. Balancing yield, B-O-4 content and

Author(s): D.S. Zylstra, A. de Santi, B. Oldenburger, J. G. de Vries, K. Barta P. J. Deuss

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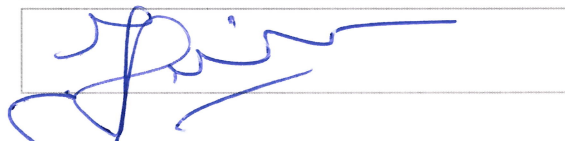
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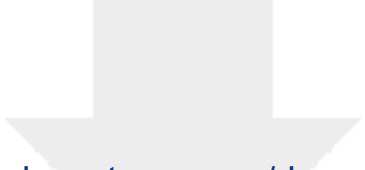
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