

Influence of hydrocracking and ionic liquid pretreatments on composition and properties of *Arabidopsis thaliana* wild type and CAD mutant lignins

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ABSTRACT

Lignin is the primary contributor to the high cost of biofuel-production from lignocellulosic biomass. In order to study lignin removal and the release of aromatic monomers, we applied hydrocracking and ionic liquid pretreatments on *Arabidopsis thaliana* biomass from both wild type (WT) and a mutant (*CAD cxd*) defective in two cinnamyl alcohol dehydrogenase genes involved in the lignin biosynthetic pathway. For *Arabidopsis* WT, our results highlight that pretreatments reduce average molecular weight of lignin by about 65% and decrease the content of β -O-4 linkages between lignin monomers. For *Arabidopsis* CAD mutant, an opposite effect is evidenced. Fewer differences were observed on depolymerization and molecular structure of lignin, which indicates that (8-O-4), (8-5), and (8-8) linkages observed in CAD mutant make lignin more resilient to pretreatment than wild-type lignin. Finally, our study shows the potential of hydrocracking pretreatment technology for extracting valuable aldehyde monomers such as vanillin and syringaldehyde from biomass.

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1. Introduction

In the techno-economic context of bioenergy and green chemicals production, the pretreatment step to remove lignin and to enable conversion of cellulose into fermentable sugars remains the key challenge in the production of inexpensive lignocellulosic biofuels and platform molecules [1]. In this way, the biorefinery field is focusing much of its attention on lignin, the largest available resource of natural aromatic polymers, but also the primary contributor to the high cost of sugar production from lignocellulosic biomass. Indeed, cellulose and hemicellulose polymers are embedded with lignin, making them highly resistant to extraction and enzymatic hydrolysis [2–4]. Lignin valorization is also very important in future biorefineries. Monomeric phenols extraction

under mild reaction conditions (hydrothermal), or with environmentally benign solvents (e.g., ionic liquids, deep eutectic solvents) is desirable [5].

Literature describes many strategies to reduce lignin content or alter lignin composition and molecular structure, with the overall goal of increasing cell wall degradability [6–9].

One of these consists in modifying the lignin biosynthetic pathway to reduce lignin content in biomass and increase accessibility of other lignocellulosic components (namely cellulose and hemicelluloses) to degrading enzymes [2]. To achieve this, genetic modifications (allelic variation and transcript reduction) involved in particular steps of the pathway are used to decrease lignin content in various plant species [10–16]. In this way, omics studies and advances in genomics and transcriptomics enable the discovery of novel enzymes to manipulate lignin content and composition [14]. Reducing total lignin is mainly achieved through down-regulation of one or more key enzymes in the phenylpropanoid pathway. Downregulating the activity of any enzymatic steps,

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starting from phenylalanine ammonia-lyase (PAL) down to CAD, often results in a reduction of lignin content (Fig. 1) [15]. However, significant decreases in total lignin are commonly associated with biomass loss and yield penalty, even under optimal growth conditions [16].

Alternatively, lignin recalcitrance can be reduced by modifying its monomeric composition and physicochemical properties. For example, coniferyl ferulate integration into lignin increases the enzymatic hydrolysis yield of cellulose and hemicelluloses fractions [17]. Moreover, recent studies showed that enrichments of 5-hydroxy-G- or S-units in lignin contribute to increase saccharification or defibering efficiencies of biomass without affecting yields and lignin content [18–21]. Furthermore, literature also reports on several plant species (i.e. maize, *sorghum bicolor* sp.) that have natural or chemically-induced mutations on genes encoding for enzymes involved in the lignin biosynthetic pathway (Fig. 1.) [22–25].

In the case of *Arabidopsis* CAD plants, two T₂DNA insertions affect two paralogous genes that encode for cinnamyl alcohol dehydrogenases (CAD) involved in the conversion of hydroxycinnamaldehydes into hydroxycinnamyl alcohol lignin precursors [26]. These mutations result in low-molecular-weight lignin, which makes the cell wall network less resilient and more susceptible to enzymatic saccharification [27].

Although some of these strategies induce a decrease in lignin content that increases the accessibility of cellulosic and hemicellulosic fractions to degradative enzymes, they also lead to deep modifications of the molecular composition and physicochemical properties of lignin. These changes could have a significant impact on the fractions and molecules obtained from biorefinery conversion processes and modify their efficiency and performance. In this study, we aimed at highlighting the impact of these changes on the effectiveness of two pre-treatment processes that were chosen for their ability to open lignocellulosic structures while minimizing environmental constraints. Objectives were to identify and compare specifically the impact of hydrothermal and ionic liquid

(IL) pretreatment technologies on the removal of lignin from *Arabidopsis* biomass of either WT or CAD mutant, as well as to assess their ability to release aromatic monomers, with a special emphasis on aromatic aldehydes considering the lignin monomeric composition in the CAD mutant.

2. Experimental

2.1. Raw material

Biomass from *Arabidopsis thaliana* (ecotype Wassilejska) wild type (WT) and CAD *cx*d (CAD) was used [26]. Plants were grown under a 16-h-light/8-h-dark photoperiod at $100 \mu\text{E m}^{-2} \text{s}^{-1}$ in Redi-Earth Plug and Seedling Mixture (Sun Gro Horticulture) supplemented with Scotts Osmocote Plus controlled release fertilizer (Hummert International) at 22 °C.

After growing until maturity and senescence, stems were harvested without leaves, siliques, and seeds, oven-dried at 50 °C overnight, and ball-milled to a fine powder using a Mixer Mill MM 400 (Retsch Inc., Newtown, PA) and stainless steel balls for 2 min at 30 s^{-1} .

2.2. Pretreatments

2.2.1. Hydrocracking pretreatment

Hydrocracking was performed in hastelloy steel pressure reactors ($6 \times 75 \text{ ml}$, 5000 multireactor system, Parr Instrument Co), equipped with magnetic stirrers and a controller system (model 4871 Modular Controller, Parr Instrument Co). All hydrocracking reactions were conducted in duplicate. 1 g of biomass was mixed with 40 ml of distilled water and heated until it reached a stable temperature of 200 °C. This set point temperature was maintained for 30 min. After pretreatment, the mixture was cooled down at room temperature and then vacuum filtered through a filter crucible (porosity 4). HPLC grade methanol was added to 2 ml of filtrate to obtain a 50% (v/v) methanol/water solution. The mixture

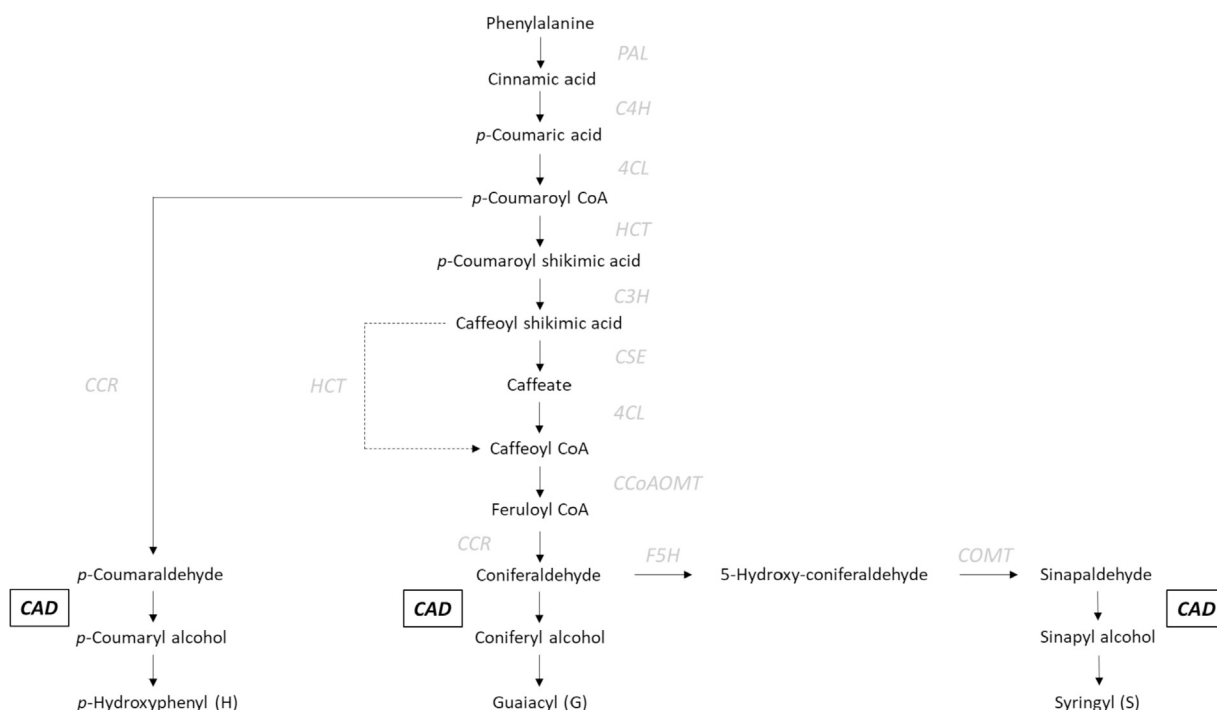


Fig. 1. Monolignol biosynthetic pathway, adapted from [26].

was then filtered using 0.2 μm PTFE syringe filters prior to LC-MS analysis. The solid recovered from the crucibles was washed with deionized water and freeze-dried (Labconco Freezone 2.5 Plus).

2.2.2. Ethyl-3-methylimidazolium acetate ([C2C1Im][OAc]) pretreatment

A 10% (w/w) slurry was prepared by mixing 1 g of biomass with 9 g of IL 1-ethyl-3-methylimidazolium acetate ([C2C1Im][OAc]) (purity: 97%) (Sigma Aldrich), in a 25 ml glass tube reactor. The reactor was heated in an oil bath at 160 °C and stirred during 3 h at 150 rpm with a magnetic stir bar. All IL pretreatments were performed in duplicate. Following pretreatments, 30 ml of deionized water were added to the slurry, with a continuous stirring, before being transferred to 50-ml Falcon tubes and centrifuged at 10,000 rpm to separate solids. Recovered pretreated biomass was washed with deionized water to remove residual IL and freeze-dried for further analyses.

2.3. Lignin extraction

Lignin extraction was performed on pretreated and raw biomass using cellulase and hemicellulase mix Cellic CTec2 and HTec2 (Novozymes S.A.). 20 μl of each enzyme mix and 30 ml of citrate buffer 0.1 N (pH 5) were added to Falcon centrifuge tubes containing 1 g of biomass finely ball-milled as previously described [7]. Tubes were placed at 50 °C for 24 h in an Enviro-Genie Incubator/Rotator system (scientific industries Inc.) and then centrifuged at 8000 rpm for 15 min to remove the enzyme mixture. This procedure was repeated four times with fresh enzyme and buffer mix. Recovered cellulolytic enzyme lignins (CEL) were washed ten times with 30 mL of water and freeze-dried (Labconco Freezone 2.5 Plus).

2.4. Free aldehydes extraction

100 mg of dried biomass were mixed with 80% (v/v) methanol/water in 1.5-ml centrifuge tubes. Tubes were placed at 70 °C at 1400 rpm in a thermomixer for 15 min and then centrifuged at 13,600 rpm for 5 min to recover the supernatant. This procedure was repeated four times. Distilled water was added to pooled supernatants to reach a 50% (v/v) methanol/water mix and filtered at 13,600 rpm using centrifugal filter (Amicon Ultracell 3 K) prior to LC-MS analysis.

2.5. Lignins characterization

2.5.1. Molecular weight distribution

CEL samples (2 mg) were placed into screw-cap glass flasks containing 2 ml of 8% acetyl bromide/92% glacial acetic acid (v/v) and incubated at 50 °C for 24 h under magnetic stirrer (250 rpm). Acetyl bromide mixture was then evaporated under nitrogen flow (DB-3D Techne sample concentrator), and samples dried at 50 °C for 1 h. 2 ml of Tetrahydrofuran (THF) containing butylated hydroxytoluene (BHT) (250 ppm) was then added to the dried residue and placed at 50 °C under stirring for 24 h. Sample were then filtered using a 0.2 μm PTFE syringe filter.

Molecular weight distribution measurements were performed by Size-Exclusion Chromatography (SEC) on a TOSOH ECOSec HLC-8320GPC separation module coupled with a SEC/MALS detector (Viscstar II Viscometer, Wyatt Technology Co) and Agilent GPC/SEC column PLgel 5 μm Mixed-D. THF containing BHT (250 ppm) was used as eluent at 1.0 mL min⁻¹.

2.5.2. Attenuated total reflectance fourier-transform infrared spectrometry analysis (ATR – FTIR)

ATR FT-IR spectra were obtained from CEL samples between

4000 cm⁻¹ and 800 cm⁻¹ with 96 scans and a resolution of 4 cm⁻¹ on a Bruker spectrometer VERTEX 70 with reflection ATR cell (Bruker Platinum diamond ATR) coupled with a room temperature HTS-XT detector, working at 10 KHz. Baseline correction and atmospheric compensation were corrected by OPUS 7.2 software algorithm.

2.5.3. Pyrolysis gas-chromatography mass spectrometry analysis (Pyro GC/MS)

Pyrolysis of CEL samples was carried out using a CDS Pyroprobe 5000 pyrolyzer (CDS Analytical Inc. City, Country). Prior to the experiments, quartz tubes were filled with 1 mg of CEL and quartz wool. CEL lignin pyrolysis occurred at a temperature of 500 °C for 30 s. The pyrolysis vapors migrated via helium gas stream (1.0 mL min⁻¹) into a GC/MS (Thermoquest Trace GC-2000/Polaris Q Mass Spectrometer, Thermoquest Co). GC injector was set at 300 °C and separation was performed using an Agilent DB-5HT (10 m \times 0.32 mm \times 0.10 μm) capillary column held first at 50 °C for 2 min, then increased to 300 °C with a heating rate of 5 °C min⁻¹, and held at 300 °C for 2 min.

2.5.4. 2D¹³C–¹H heteronuclear single quantum coherence (HSQC) nuclear magnetic resonance (NMR) spectroscopy

NMR spectroscopy analyses were performed following the protocol described by Eudes et al. [28]. CEL samples were placed in DMSO-d₆/pyridine-d₅ mix (4:1) and sonicated in a Branson 2510 table-top cleaner (Branson Ultrasonic Corporation, Danbury, CT). The homogeneous solutions were transferred to NMR tubes. HSQC spectra were acquired at 25 °C using a Bruker Avance-600 MHz instrument equipped with a 5 mm inverse-gradient ¹H/¹³C cryoprobe using a hsqcetgpsisp2.2 pulse program (ns = 400, ds = 16, number of increments = 256, d₁ = 1.0 s). Chemical shifts were referenced to the central DMSO peak ($\delta_{\text{C}}/\delta_{\text{H}}$ 39.5/2.5 ppm).

2.6. Aldehydes quantification

An Agilent 1200 series/6210 TOF LC/MS was used to perform free aldehydes quantification as previously described [29]. Free aldehydes were separated on a Poroshell-120 column with 0.1% v/v formic acid in water (solvent A) and 0.1% formic acid in acetonitrile/water (98/2, v/v) (solvent B). Elution gradient, flow rate 0.55 mL min⁻¹, was 87% A (0–5 min), 50% A (5–7 min), 87% A (8–11 min).

Mass spectrometer (MS) analysis was conducted using electrospray ionization (ESI) in positive ion mode. [M + H]⁺ detection was conducted at 0.85 spectra/s and a cycle time of 1.176 s/spectrum in full scan mode with the following settings: capillary voltage 3500 V, fragmentary 165 V, skimmer 50 V, OCT RF 170 V, nitrogen flow rate 9 L min⁻¹, nebulizer pressure 1.03 bar and 325 °C for drying gas temperature.

Calibration curves obtained from authentic compound standards (Sigma-Aldrich) were used for quantification of free aldehydes.

3. Results and discussion

Results obtained during this study highlight the impact of hydrocracking and IL pretreatment processes applied on biomass from *Arabidopsis* WT and CAD mutant. Effects of pretreatments on the macromolecular structure of lignin (molecular weight, G/H/S unit ratio and inter-unit bonds) are initially presented. Furthermore, yields of aldehyde monomers are established and the extraction potential of hydrocracking and ILs processes was determined.

3.1. Effects of hydrocracking and IL pretreatments on lignin macromolecular structure

3.1.1. Molecular weight distribution

Elution profiles obtained from CEL fractions isolated from unpretreated biomass of *Arabidopsis* WT and CAD mutant reveal significant differences regarding the molecular weight distribution (Fig. 2). For CAD mutant lignin, results show that the largest mass peak detected (>5000 Da) was significantly reduced and that smaller molecular mass fractions (<2000 Da) were more abundant. Number average molecular weight (M_n) (4780 Da (WT) vs 960 Da (CAD)), weight average molecular weight (M_w) (22,400 Da (WT) vs 2990 Da (CAD)), polydispersity index (D) (4.7 (WT) vs 3.1 (CAD)) and statistical median of the distribution (3370 Da (WT) vs 750 Da (CAD)) also indicate that CAD lignin has a lower molecular weight than WT (Table 1).

These elution profiles and M_n , M_w , D and statistical median values, which are consistent with previous investigations [27] using *Arabidopsis* WT and CAD mutant, result from the lack of CAD enzymes in the mutant, which are involved in the synthesis of hydroxycinnamyl alcohols, the conventional lignin monomer precursors involved in the polymerization process [24,30]. These results indicate that biomass from CAD mutant crops could be very effective for biorefinery processes, considering that shorter lignins prevent the formation of a recalcitrant lignin network within the biomass and increase the accessibility of degrading enzymes to polysaccharidic fractions [31].

Regarding pretreatment effects, M_n , M_w , D and statistical median values obtained from size exclusion chromatography of CEL fractions from pretreated biomass samples of *Arabidopsis* WT and CAD show noticeable differences (Table 1).

For WT, hydrocracking (HC, 200 °C, 30 min) and IL pretreatment ([C2C1Im][OAc], 160 °C, 180 min) induce a decrease of lignin

molecular weight (M_n WT: 4780 Da, M_n WT-HC: 1730 Da, M_n WT-IL: 1720 Da). These results are consistent with the studies conducted by Liu et al. (2017, 2019) in switchgrass, which showed a significant decrease of lignin molecular weight after IL pretreatment [32,33].

Conversely, few effects were observed for CAD mutant (M_n CAD: 960 Da, M_n CAD-HC: 1050 Da, M_n CAD-IL: 970 Da).

Although molecular weight of CAD lignin is initially much lower (M_n WT: 4780 Da vs M_n CAD: 960 Da), results obtained from hydrocracking and IL pretreatments indicate that CAD lignin was more resistant to these treatments. These observations could reflect the modifications occurring during lignin synthesis in the CAD mutant. Disruption of CAD genes results in the incorporation of non-conventional hydroxycinnamaldehydes in lignin, which alters units proportion and lignin molecular structure [34].

To confirm this hypothesis, G/H/S unit composition, 2D 13C–1H HSQC NMR and FT-IR measurements were performed on CEL obtained from WT and CAD mutant before and after biomass pretreatments.

3.1.2. Lignin G/H/S composition

Determination of lignin G/H/S composition of *Arabidopsis* WT (made from alcohols) and CAD mutant (made from aldehydes) with and without pretreatment using pyrolysis GC/MS is summarized in Table 2.

Arabidopsis WT presents a G/H/S composition of 40:1:25, which corresponds to a S/G ratio of 0.6. For CAD mutant, proportions obtained show a very high increase of H units (from 1.5% in WT to 12.8%) combined with a decrease of G units (from 61.1% in WT to 44.4%) and similar S-unit contents (from 37.4% in WT to 40.8%). This important increase of H-units could lead to a more reticulated lignin that is more recalcitrant to depolymerization. Indeed, increased H units could lead to increased condensed linkages (e.g.,

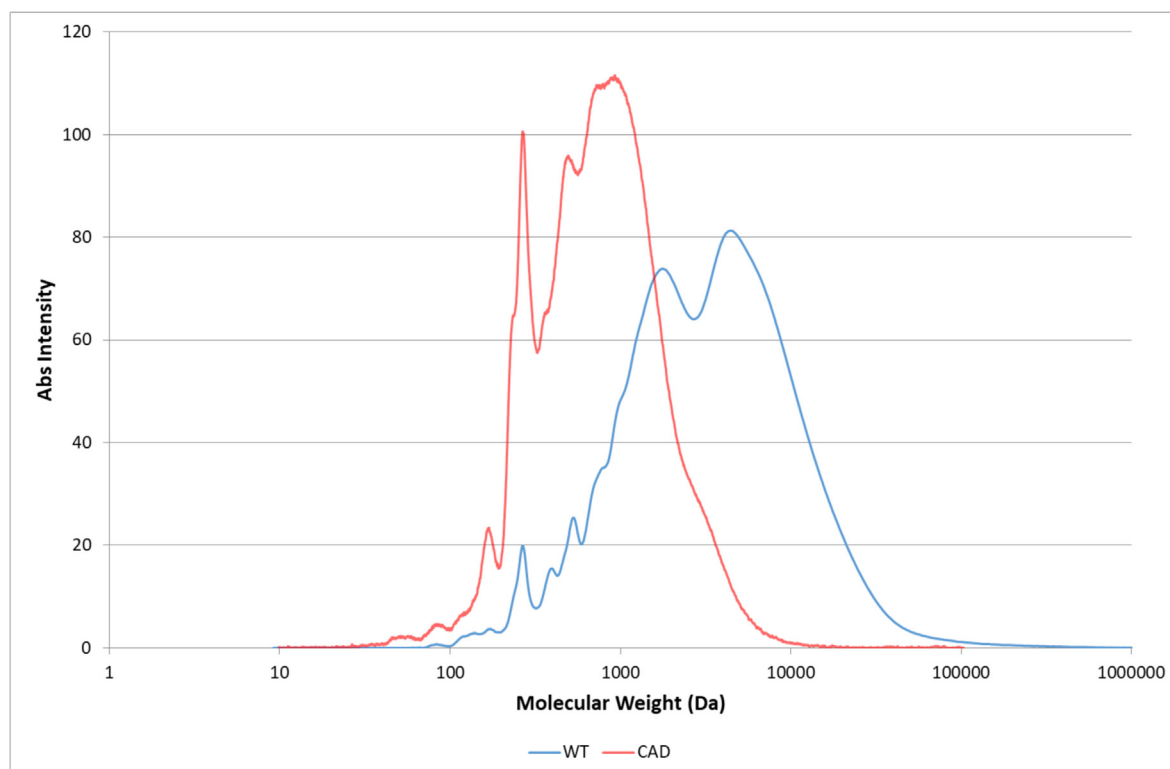


Fig. 2. Molecular weight distribution of CEL fractions from *Arabidopsis* WT and CAD mutant.

Table 1

Effects of hydrocracking (HC) and ionic liquid (IL) pretreatments on number average molecular weight (M_n), weight average molecular weight (M_w), polydispersity index (D) and median value of CEL fractions from Arabidopsis WT and CAD mutant.

	WT ^a	WT-HC ^b	WT-IL ^c	CAD ^d	CAD-HC ^e	CAD-IL ^f
M_n = number average molecular weight (Da)	4780	1730	1720	960	1050	970
M_w = weight average molecular weight (Da)	22,400	5020	3720	2990	2870	2870
D = dispersity index	4.7	2.9	2.2	3.1	2.7	3.0
D(0.5) = median (Da)	3370	1115	1304	750	720	700

^a WT CEL.^b WT CEL hydrocracking pretreatment.^c WT CEL IL pretreatment.^d CAD CEL.^e CAD CEL hydrocracking pretreatment.^f CAD CEL IL pretreatment.**Table 2**

Pretreatment effect on G/H/S monomer composition of lignin from Arabidopsis wild-type and CAD mutant.

	WT ^a	WT-HC ^b	WT-IL ^c	CAD ^d	CAD-HC ^e	CAD-IL ^f
H-units (%)	1.5	4.5	0.7	12.8	13.5	14.5
G-units (%)	61.1	59.3	64.3	46.4	44.4	45.0
S-units (%)	37.4	36.1	35.0	40.8	42.1	40.4

^a WT CEL.^b WT CEL hydrocracking pretreatment.^c WT CEL IL pretreatment.^d CAD CEL.^e CAD CEL hydrocracking pretreatment.^f CAD CEL IL pretreatment.

β -5 bonding) within the polymer due to the relative decrease of 3,5-dimethoxylated aromatics that promote β -O-4 linkages during lignin polymerization [35,36].

G/H/S composition analysis showed that hydrocracking and IL pretreatments have few impacts on G/H/S unit proportion. For WT, G (WT: 61.1%, WT-HC: 59.3%, WT-IL: 64.3%) and S (WT: 37.4%, WT-HC: 36.1%, WT-IL: 35.0%) units proportion stay similar. A slight increase of H unit is observed after hydrocracking (WT: 1.5%, WT-HC: 4.5%, WT-IL: 0.7%). For CAD mutant, pretreatments have few effects on G (CAD: 46.4%, CAD-HC: 44.4%, CAD-IL: 45.0%), S (CAD: 40.8%, CAD-HC: 42.1%, CAD-IL: 40.4%) and H (CAD: 12.8%, CAD-HC: 13.5%, CAD-IL: 14.5%) unit proportions and therefore do not modify the monomeric composition of non-conventional aldehyde lignin units.

3.1.3. FT-IR analysis

FT-IR analyses performed on untreated lignin from Arabidopsis WT and CAD mutant are presented in Fig. 3.

First, profiles obtained show a reduction of intensity at 1030 cm^{-1} for the mutant. This decrease could be correlated to several modifications of the lignin structure: A decrease of alcohol monomers results in a decrease of C–O bonds linked to primary and secondary alcohols, which are mainly measure at 1030 cm^{-1} [37]. Moreover, a decrease of alcohol units also reduces the presence β -O-4 linkages (C–O–C, β -aryl ether), which have a maximum at 1030 cm^{-1} [38]. The lower intensity of peaks related to this type of bonding indicates the presence of other forms of chemical bonds in the lignin structure. Further, G/H/S determination showed an increase of H units, which correlates with a decrease of 3,5-dimethoxylated aromatics. This decrease influences the amount of C–O bonds present in the lignin structure and could also contribute to the intensity decrease observed at 1030 cm^{-1} .

A second major difference is observed for intensity around 1630 cm^{-1} . Several studies showed that skeletal ring vibration and C=O stretch present in various aldehyde monomers have a maximum at 1610 cm^{-1} [22,38,39]. This observation is consistent

with the mutant's lignin which mainly consists of aldehyde monomers.

After pretreatment, FTIR profiles show a slight decrease at 1030 cm^{-1} , related to C–O stretch for WT lignin (Fig. 4.), combined to an increase in the carbonyl groups (also suggested by relative increase at 1630 cm^{-1}). These results could be explained by a decrease of β -O-4 bonds due to lignin depolymerization during the pretreatment [40].

For the mutant (Fig. 5), FTIR spectra of lignin showed similar profiles for both non-pretreated and pretreated biomass samples. These results, in line with molecular weight analysis and G/H/S unit determination, confirm the low impact of hydrocracking and IL pretreatments on the structure of CAD lignin.

3.1.4. $2D^{13}\text{C}$ – ^1H heteronuclear single quantum coherence (HSQC) NMR spectroscopy

$2D^{13}\text{C}$ – ^1H HQSC NMR spectroscopy was used to characterize lignins extracted from Arabidopsis WT and CAD mutant. Regarding WT, results show that lignin is composed of typical G and S units and presence of aldehyde units is minimal. Typical signals from β -aryl ether (A), phenylcoumaran (B), and resinol (C) inter-linkages were readily visible (Fig. 6). Analysis of aromatic regions of CAD mutant shows very few conventional G and S units derived from hydroxycinnamyl alcohols. Main signals correspond to unusual guaiacyl (G') and syringyl (S') units derived from the polymerization of hydroxycinnamaldehydes (Fig. 6). Similarly, no conventional inter unit linkages (β -aryl ether (A), phenylcoumaran (B), and resinol (C)) are observed in the CAD mutant aliphatic region of the spectra, but instead, non-conventional A' (8-O-4), B' (8-5), and C' (8-8) linkages from aldehydes were observed in the aldehyde region of the spectra (Fig. 6).

These results, which confirm that chemical bonds established between aldehyde units in the CAD mutant are different from those in the WT, are consistent with observations made by Zhao et al. (2013) and Kim et al. (2019) on CAD mutants, showing that lignin is derived almost exclusively from coniferaldehyde and sinapaldehyde units linked together by non-conventional 8-O-4, 8-5, 8-8 chemical bonds [5,41].

This striking chemical linkage difference from classical lignin could also be correlated to the changes observed for the molecular weight distribution. Although mutant lignin, have a lower initial polymerization level, they are more difficult to depolymerize by pretreatment processes such as hydrocracking or IL. This is possibly due to (8-O-4), (8-5), and (8-8) linkages which seem more difficult to break than classical chemical bonds.

3.1.5. Influence of hydrocracking and IL pretreatments on aldehyde extraction

In parallel with the identification of pretreatment effect on physicochemical properties of CEL fractions obtained from

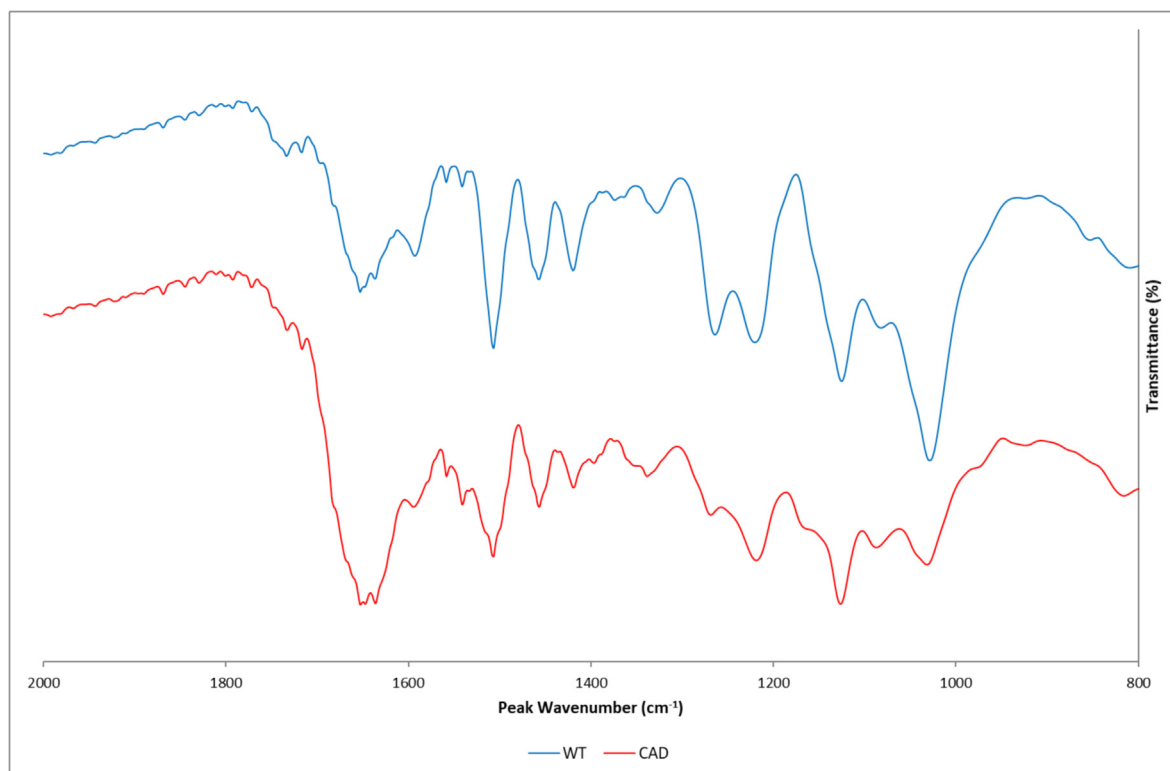


Fig. 3. Infrared spectrometry (FTIR) of CEL fractions from Arabidopsis WT and CAD mutant.

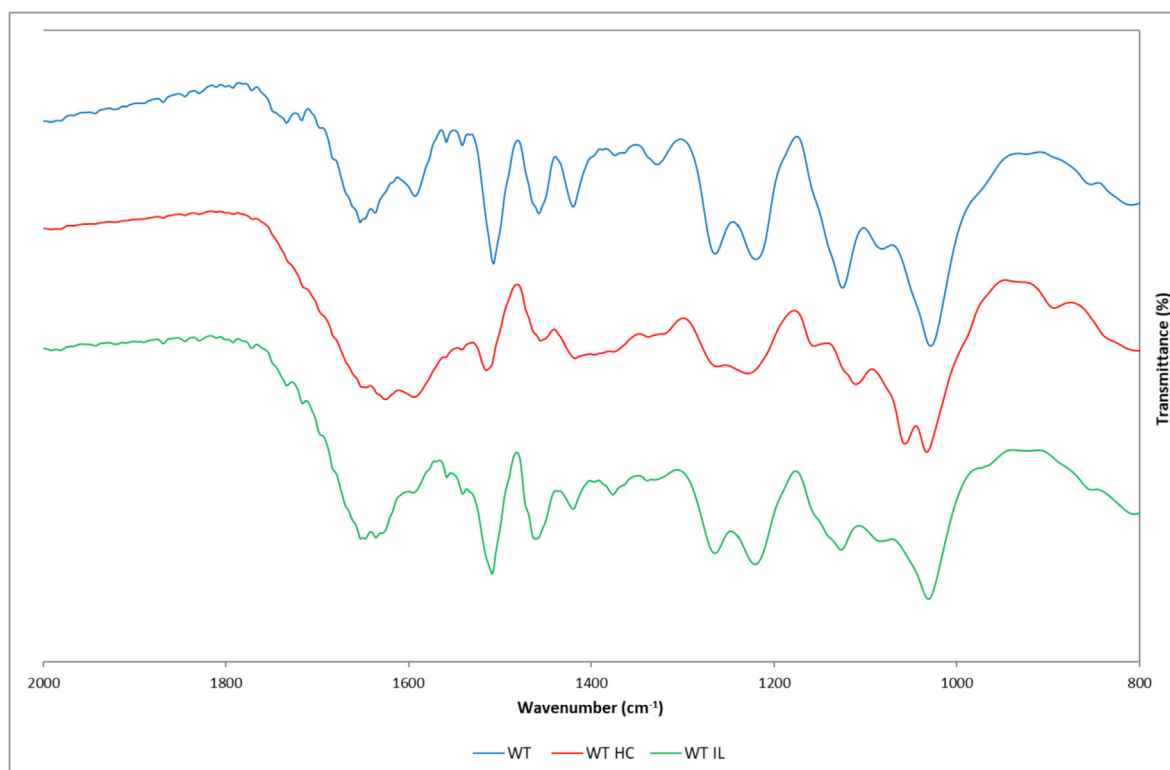


Fig. 4. Infrared spectrometry (FTIR) of CEL fraction from Arabidopsis WT after biomass pretreatments (HC: hydrocracking, IL: ionic liquid).

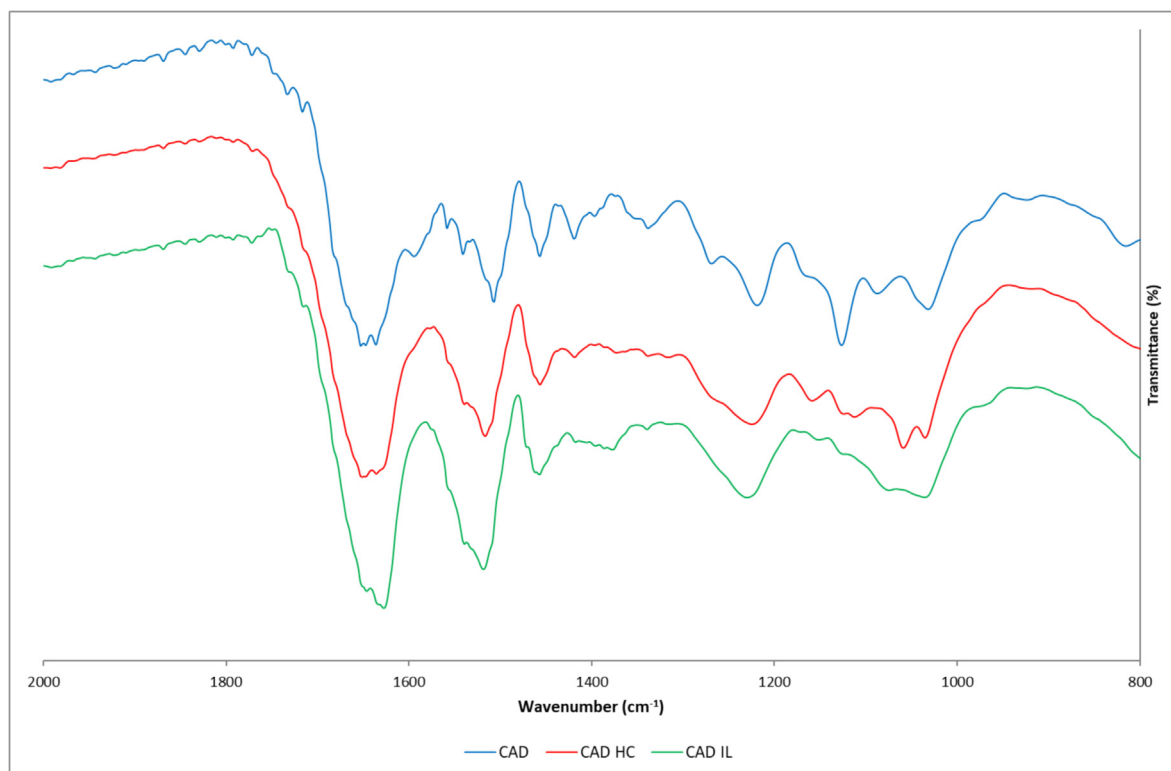


Fig. 5. Infrared spectrometry (FTIR) of CEL fractions from Arabidopsis CAD mutant after biomass pretreatments (HC: hydrocracking, IL: ionic liquid).

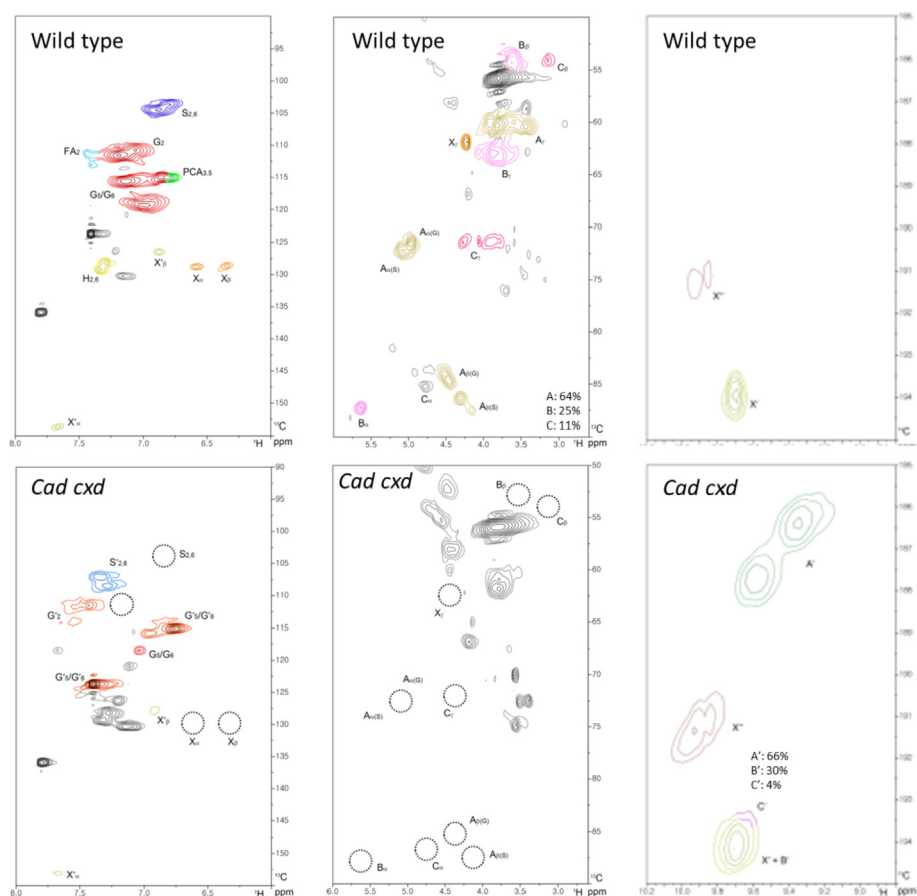


Fig. 6. Partial short-range ^{13}C – ^1H (HSQC) spectra of CEL fractions purified from untreated Arabidopsis biomass from wild type (upper panels) and CAD mutant (lower panels). Left panels: aromatic region. Middle panels: aliphatic region. Right panels: aldehydes region.

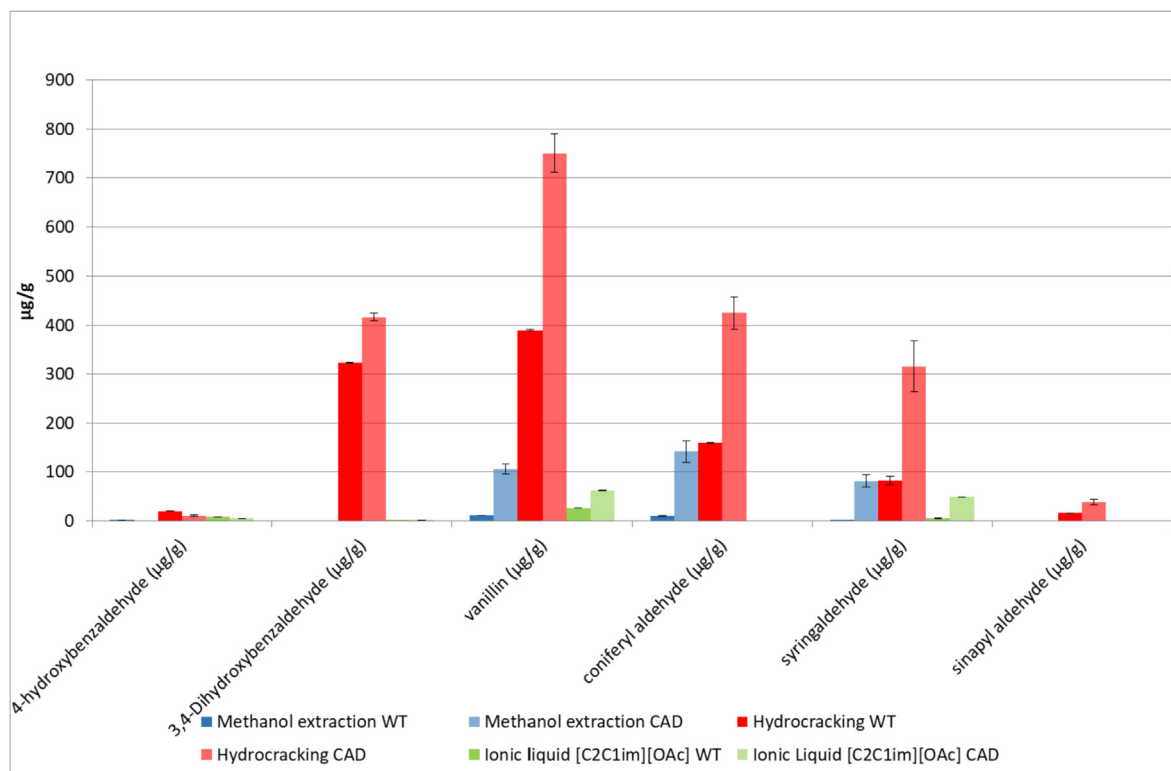


Fig. 7. Determination of aldehydes extracted from biomass of *Arabidopsis* WT and CAD mutant after hydrocracking and IL pretreatments.

Arabidopsis WT and CAD mutant, the yield of aldehydes extracted upon pretreatments was determined (Fig. 7).

First, results show that methanol extracts obtained from untreated CAD biomass exhibit a higher content of free aldehydes than those from untreated WT (vanillin: WT = 11 µg/g vs CAD = 106 µg/g; coniferyl aldehyde: WT = 10 µg/g vs CAD = 141 µg/g; syringaldehyde: WT = 1 µg/g vs CAD = 81 µg/g). This result appears to be consistent with the disrupted monolignol biosynthetic pathway occurring in the mutant.

After pretreatment, results show that hydrocracking increases the extraction efficiency of aldehydes for both WT and CAD mutant compared to the methanol-only extraction method (Fig. 7). In particular, higher amounts of vanillin (WT = 390 µg/g vs CAD = 750 µg/g), coniferyl aldehyde (WT = 160 µg/g vs CAD = 425 µg/g), syringaldehyde (WT = 82 µg/g vs CAD = 315 µg/g), and 3,4-dihydroxybenzaldehyde (WT = 320 µg/g vs CAD = 420 µg/g) were obtained in the case of CAD mutant. Hydrocracking technology could therefore be a selective method to obtain specific valuable aromatic compounds (e.g. vanillin) from inexpensive various industrial by-products and waste (i.e., paper industry) [42].

Conversely, poor yields of aldehyde monomers were observed after IL pretreatment (Fig. 7). A first hypothesis to explain the absence of free aldehydes would be related to their oxidation. Howarth showed that several aromatic aldehydes become oxidized when using the catalyst [Ni(acac)₂] and dioxygen in presence of 1-ethyl-3-methylimidazolium [43]. When the reactions were carried out without catalyst, authors showed that carboxylic acids could still be formed, but with significantly lower yields. Considering the pretreatment time and the presence of oxygen during IL pretreatment, an oxidation of aldehyde monomers after their release could have occurred. Furthermore, a second hypothesis to explain the absence of free aldehydes relates to their high solubilities and

affinities to IL, which could make their separation from IL difficult and explain the lack of detection.

4. Conclusion

In this study, hydrocracking and ([C2C1Im][OAc]) IL pretreatments were applied to biomass from *Arabidopsis* WT and CAD mutant. Results show that pretreatments have a significant influence on WT lignin depolymerization but fewer effects on the CAD lignin. FTIR and 2D ¹³C–¹H HSC NMR analyses revealed significant differences in the types of lignin chemical bonds between the two genotypes. Regarding these lignin chemical linkages, our study shows that WT lignin are predominantly composed of β-O-4 bonds compared to the mutant lignin for which the preponderance of aldehyde units favors other types of chemical bonds.

Concerning the IL process, our results highlight the higher potential of hydrocracking pretreatment technology for the extraction of aldehydes from the mutant biomass, and its ability to yield specific valuable aromatic compounds such as vanillin, syringaldehyde, coniferyl aldehyde and protocatechuic aldehyde. Furthermore, the yield of aldehyde monomers is higher when the pretreatment technologies are applied to CAD mutant biomass.

Finally, it appears, for this particular case that lignin from CAD mutant plants is much more resistant than WT lignin. This feature suggests that this type of aldehyde-rich lignin could be considered for the manufacturing of more resistant biocomposite materials.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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