

# Effect of steam explosion treatment on chemical composition and characteristic of organosolv fescue lignin



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## ABSTRACT

Tall fescue, a grass currently grown as forage, presents a good potential as a feedstock in the development of biorefining. In this study, combination of steam explosion and an acetic/formic acid “organosolv” pretreatments are applied on raw fescue to optimize lignin extraction yields and obtain specific products. In a first step, fescue is pretreated by steam explosion under severity factors from 1.4 to 4.2. These treatments allow hemicelluloses solubilization and open the lignocellulosic structure. Steam exploded solid residues are then treated with an acetic acid (50%)/formic acid (30%)/water (20%) mixture. Extracted lignins are precipitated and characterized by size exclusion chromatography (SEC), <sup>31</sup>P NMR and HSQC NMR. Results show that acetic and formic acids extracted lignins present G, H and S units with ferulate and *p*-coumarate. Lignin typical linkages identified are of  $\beta$ -O-4 and spirodienone substructures. Combination of steam explosion and acetic and formic acid pretreatments increases extraction yields from 30% to 100%. Moreover, an increase of the steam explosion treatment intensity induces chemical modifications in lignin structure such  $\beta$ -O-4 and spirodienone substructure degradations, increase of free COOH and phenolic OH bonds, decrease of aliphatic OH ferulate and *p*-coumarate bonds and changes in G/H/S units proportions. Steam explosion intensity affects also lignin molecular weights.

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## 1. Introduction

Tall fescue, a pluriannual *Poaceae* family plant, is currently used for animal feed and presents a good potential for the second-generation biorefining development. Fescue grows typically in Northern Europe from March to October with a productivity of six to fifteen tons of dry matter by ha. This crop possesses a long vegetation season, a ground cover in winter and an easy culture and harvest which requires less of plant protection agents (Besnard et al., 2013).

Lignin is the second most abundant natural polymer in the world behind cellulose. Its occurrence and relative amount in biomass depends mainly on plant species. Currently, lignin interest increase in Europe as high-added value materials for specific applications and formulations (Azadi et al., 2013; Rochez et al., 2013; Thakur and Thakur, 2015).

Lignin is formed by oxidative biopolymerization of three alcohol-containing monomers (called monolignols): synapyl alcohol, *p*-coumaryl alcohol and hydroxyphenyl alcohol which account respectively syringyl units (S), guaiacyl units (G) and *p*-hydroxyphenyl units (H). Lignin presents a complex structure with irregular and specific ether or carbon-carbon bonds including mostly  $\beta$ -O-4,  $\beta$ -1,  $\beta$ - $\beta$ , 4-O-5, 5-5,  $\beta$ -5. Units and bonds proportions vary according to plant species, plant origins or agronomic parameters such as harvesting time. In lignocellulosic structure, lignin is moreover linked to sugar polymers (cellulose and hemicelluloses) with ether and ester bonds creating a “lignin-carbohydrates complex” (Manara et al., 2014; Vanderghem et al., 2012; Zikeli et al., 2016).

Different processes are able to partially break lignin bonds in lignocellulosic structures. Chemical pretreatments are particularly effective for lignin extraction/recovery. Commonly, mineral or organic acid and bases and/or organic solvents (organosolv process) are used alone or in combination. In the specific case of organosolv pretreatments, methanol, phenol, 1,4-dioxane, ethanol, acetic acid, acetic acid/formic acid are the most convenient organic solvents for industrial pretreatments process. Organic acids give good separa-

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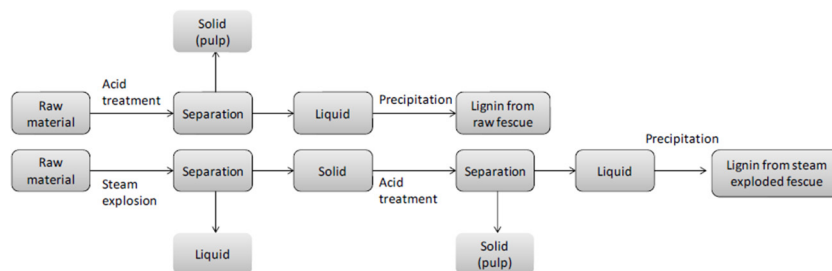


Fig. 1. Pretreatment diagram of tall fescue.

tion yields between carbohydrates and lignin with different effects on lignocellulosic structure, the hydrogen ions, formed during dissolution, increase the delignification, the hemicelluloses hydrolysis and lignin solubilization (Delmas, 2011; Vanderghem et al., 2012).

In another way, literature shows that steam explosion technology impacts lignin structure (Balat 2011; Kataria et al., 2016). Steam explosion is a thermophysico-chemical process which provides mechanical destructuration of lignocellulosic material by a combination of two steps: vapocacking and explosive decompression. Chemical reactions are initiated by thermo-hydrolyzed of biomass (Duque et al., 2016; Jacquet et al., 2015; Kataria et al., 2016; Sun and Cheng, 2002).

This technology does not require any chemical products for the pretreatment. The overall process carbon footprint depends on the heat source used to produce steam and recycling of vapor generated. Steam explosion biomass pretreatment for the second-generation biorefinery is currently in industrial prospect. Future technical challenges aim to obtain a continuous or semi-continuous process in high scale with a minimum of investment and an optimum energetic efficiency.

The purpose of the study is to combine the steam explosion low environmental impact technology with organosolv process (acetic acid/formic acid pretreatment) to increase significantly lignin extraction yield and obtain specific product. Results obtained show effects of this pretreatment combination on lignin extraction yields and lignin structural properties and take place in a context of global biorefining of lignocellulosic material.

## 2. Materials and methods

### 2.1. Raw material

Tall fescue (*Festuca arundinacea*) was cultivated in Belgium by Walloon Agricultural Research Center in Gembloux. Material was previously dried (three days at 60 °C) and milled to reach a particle size of 0.75 mm.

### 2.2. Steam explosion process

Steam explosion pretreatment was carried out in prototype pilot scale equipment with a 50 L reactor volume (Jacquet et al., 2011, 2012). This prototype consists in a steam generator (29.4 kW, operating pressure: 6.0 MPa), a 50 L vessel designed to reach a maximum operating pressure of 5.1 MPa and an explosion tank, in which the product is recovered. A quick-opening ball valve, placed between the vessel and the explosion tank, is used to decrease quickly the pressure and gives the explosion effect. Steam explosion pretreatments were carried out on 1 kg of tall fescue (91.4% of dry matter). After placing the material in the reactor, combination of steam pressure and retention time was adjusted to obtain a severity factor ranging from 1.4 to 4.2 (Fig. 2). This range was selected to preserve cellulose from thermal degradations as observed by Jacquet et al., (2011). As described further in Fig. 1, pretreated material was cen-

trifuged in a Rousset RC40VxR (18 L bowl volume) at 3000 rpm to separate solid from liquid. Severity factor are calculated with the relation developed by Jacquet et al. which obeys to Arrhenius law (kinetics of first order):

$$S = \log_{10} \sum \frac{14,75(t_{n+1} - t_n)}{(T_{n+1} - T_n)} \left[ \exp\left(\frac{T_{n+1} - 100}{14,75}\right) - \exp\left(\frac{T_n - 100}{14,75}\right) \right]$$

Where:  $S$  = severity factor,  $t$  = process time (s) and  $T$  = process temperature (°C). Time needed to reach the desired temperature is also taken into account (Jacquet et al., 2011).

### 2.3. Organosolv process and lignin purification

Raw tall fescue and steam exploded tall fescue were pretreated according to the protocol described in Vanderghem et al. (2012) with some adaptations (Fig. 1). Pretreatments were performed in 500 mL double-necked boiling flasks at 107 °C in a formic acid/acetic acid/water mixture of 50/30/20 (% volume ratio) for 3 h at a liquid/dry matter ratio of 25/1. During cooking, stirring was continuously applied at 400 rpm. After the reaction, media were vacuum filtered on a fritted disk (40–100 μm pore size; Robu Glasfilter-Geräte GmbH) to recover solid and liquid fractions (“pulp” and “black liquor”). Pulps were then washed twice with the same cooking mixture (added to black liquor) and distilled water (2 L). Pulp yield after pretreatment was determined gravimetrically after drying at 50 °C to constant weight. Black liquor (containing mostly the lignin) was diluted 1/10 in water and pH was adjusted to 2.0 in order to precipitate lignin, which were recovered by centrifugation (Beckman Coulter Avanti J-E, 8000g, 10 min) and freeze-dried.

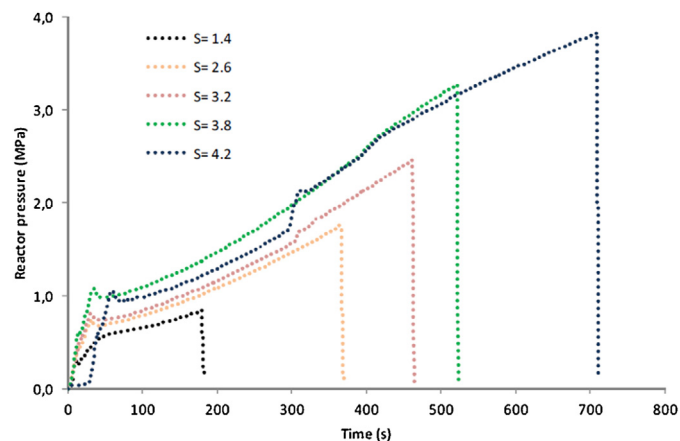


Fig. 2. Reactor pressure during steam explosion pretreatment under different severity factors (S).

## 2.4. Compositional analysis

Proteins, extractives, total lignin, ash content and monosaccharides contents of raw tall fescue were determined. Ash content was determined by combustion of the samples at 525 °C for 4 h (Sluiter et al., 2005a). Proteins content was determined by Kjeldahl procedure using a conversion factor of 6.25. For extractives determination, samples were successively extracted at ebullition temperature with water and ethanol in a Soxhlet extractor (Sluiter et al., 2005b).

Total lignin, soluble lignin and insoluble lignin content were assessed gravimetrically using the Klason methodology where raw samples were hydrolyzed with 72% sulphuric acid (30 °C for 60 min), followed by dilution to 4% sulphuric acid with distilled water and hydrolyzed in an autoclave (121 °C for 60 min). The mixture was filtered through a filtering crucible (P4) previously ignited and tarred, dried at 105 °C to a constant weight and ignited in a muffle furnace at 525 °C for about 18 h giving Klason lignin content estimated gravimetrically (Sluiter et al., 2008). Acid Soluble Lignin (ASL) was measured on filtrate by UV absorption at 205 nm. Total lignin content was then obtained by the sum of Klason lignin and acid soluble lignin (Ehrman, 1996).

Cellulosic glucose composition was determined after 1 h of pre-hydrolysis of samples in 72% sulphuric acid at 30 °C followed by 6 h of hydrolysis at 100 °C in 1 M sulphuric acid. Non-cellulosic sugars were determined after 3 h of hydrolysis in 1 M sulphuric acid. Monosaccharides were determined as alditol acetates by gas chromatography. Reduction of monosaccharides and acetylation was performed according to the procedure described by Blakeney et al. (1983). Analyses were carried out with a Hewlett-Packard (HP1 7890B) gas chromatograph equipped with a flame ionization detector. Components were separated using high performance capillary column, HP1-methylsiloxane (30 m × 0.32 mm, 0.25 μm, Scientific Glass Engineering, S.G.E. Pty. Ltd., Melbourne, Australia). Data were analyzed using ChemHP software. 2-Deoxyglucose (internal standard), glucose, xylose, arabinose, mannose and galactose, used as standards, were obtained from Sigma-Aldrich (St. Louis, USA). All analyses were performed at least in duplicate.

## 2.5. Lignin characterization

### 2.5.1. Size exclusion chromatography

Molecular mass distribution of lignin was determined by size-exclusion chromatography (SEC) according to the method provided by the International Lignin Institute (ILI008 – version 2.4). Waters 2690 Alliance chromatograph coupled to a photodiode assay detector Waters 996 and equipped with a TSK-Gel PWxl guard column and two TSKgel G3000PWXL columns (300 mm × 7.8 mm I.D.) in series (with respectively 7 and then 6 μm particle diameter) (Tosoh Bioscience, Tessenderlo, Belgium) was used. Polynomial calibration curve was established by using a range of sodium polystyrene sulfonates (Na-PSS) (Sigma-Aldrich, Diegem, Belgium), at a concentration level of 1 mg/mL: Weight-average molecular mass ( $M_w$ ) were equal to 208, 4300, 6780, 10600, 16800 and 32000 Da. Eluent solution was composed of NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O (0.35 M) and NaOH in pellet form (0.08 M), completed to 1 L with distilled water. After complete dissolution, the solution was adjusted to pH 12.0 with 0.5 M NaOH and filtered through glass filter (0.45 μm). The flow rate was adjusted at 0.9 mL/min and temperature at 30 °C. Detection was performed at 280 nm.

Lignin samples were dissolved in a phosphate buffer eluent solution, at a concentration of 3 mg/mL under gentle stirring for at least 48 h, and filtered through a 0.45 μm filter prior to injection.

$M_w$  and number-average molecular mass ( $M_n$ ) were calculated from the raw data of UV Abs<sub>280</sub> as a function of elution volume, with ( $C_i$ ) solute concentration of mass  $i$  according to:

$$M_w = \frac{\sum(C_i * M_{wi})}{\sum C_i} \quad \text{and} \quad M_n = \frac{\sum C_i}{\sum(C_i/M_{wi})}$$

With  $M_{wi}$  calculated with the polynomial equation previously established with standards (Na-PSS). The ratio  $M_w/M_n$  represents the polydispersity of the samples.

### 2.5.2. NMR spectroscopy

Nuclear magnetic resonance (NMR) analyses were performed using a Varian Unity 600 MHz instrument at 298 K. 50 mg of lignin were dissolved in 0.75 mL DMSO-*d*<sub>6</sub> (euriso-top). <sup>1</sup>H chemical shifts were referenced to Tetramethylsilane (δ 0.0 ppm). Adiabatic broadband {<sup>13</sup>C-<sup>1</sup>H} 2D heteronuclear (multiplicity edited) single quantum coherence (g-HSQCAD) analyses were performed applying a Varian ChemPack 4.0 pulse sequence with 2048 collected complex points for the <sup>1</sup>H dimension and a relaxation delay of 5 s. Number of transients was 32.256 time increments were recorded in the <sup>13</sup>C dimension. The <sup>1</sup>J<sub>C-H</sub> used was 140 Hz.

Quantitative <sup>31</sup>P NMR analysis of lignin was performed according to a protocol adapted from Pu et al. (2011). 20 mg of lignin was dissolved in 450 μL of a solution of anhydrous pyridine (Sigma-Aldrich) and deuterated chloroform (euriso-top) (1.6:1.0, v/v) containing 11.5 mg of chromium(III) acetylacetonate (Sigma-Aldrich), as relaxation agent, and 12 mg of cyclohexanol as internal standard. Lignin derivatization was performed by adding 100 μL of 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (TMDP) (Sigma-Aldrich).

Spectrum was acquired using a Varian VNMRs 600 MHz spectrometer, with 90° pulse angle, relaxation delay of 25 s, acquisition time of 1.049 s and accumulation of 1600 scans. NMR data were processed using Varian VNMRJ Software. All chemical shifts were reported relative to the cyclohexanol signal (Merck) (144.9 ppm). The content of hydroxyl groups was obtained by integration of the following spectral regions: aliphatic hydroxyls (148.6–146.0 ppm), *p*-hydroxyphenyl (H) phenolic hydroxyls (138.4–137.6 ppm), and carboxylic acids (135.4–134.0 ppm). 5-substituted and G-non-condensed phenolics were estimated by the integration of the zone comprise between 143.6 and 138.6 ppm.

## 3. Results and discussion

### 3.1. Raw fescue composition

Results reported in Table 1 show that dry raw fescue is composed by cellulose (33.8 ± 2.8%), hemicelluloses (26.0 ± 0.1%), total lignin (16.6 ± 0.5%), proteins (6.6 ± 0.0%), ash (8.3 ± 0.0%) and some recovered in extractives (17.9 ± 0.3% (water) and 2.6 ± 0.2% (ethanol)).

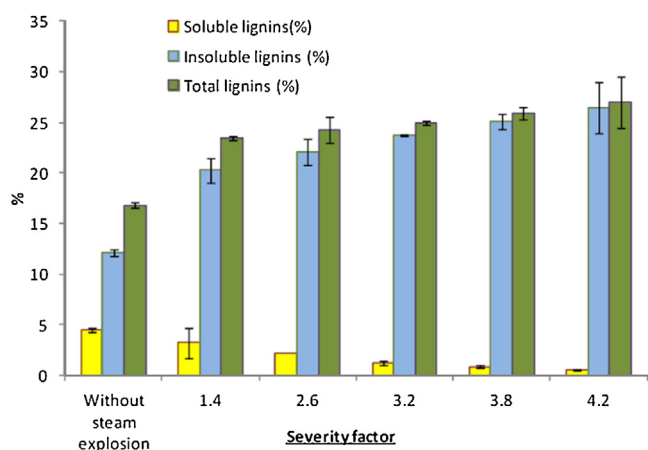
Hemicelluloses are composed of 17.2 ± 0.0% xylose, 4.0 ± 0.0% glucose, 2.8 ± 0.0% arabinose, 1.2 ± 0.0% galactose, 0.6 ± 0.0% mannose and 0.2 ± 0.1% rhamnose. These results suggest that hemicellulosic structure in fescue is mainly xylan- and arabinoxylan-type and eventually xyloglucan. Total lignin is composed of Acid Soluble Lignin (ASL) 4.5 ± 0.2% and Acid Insoluble Lignin (Klason) 12.1 ± 0.3%. High dry matter yield of raw fescue, combined to high sugars content, place raw fescue as good feedstock for biorefinery purpose.

### 3.2. Raw tall fescue characterization after steam explosion pretreatment

For Severy Factor ( $S < 2.6$ ), results obtained show that total lignin content in the solid increases with steam explosion pre-

**Table 1**  
Raw fescue composition expressed in% of dry matter.

		Dry matter%
Lignin		16.6 ± 0.5
	Soluble	4.5 ± 0.2
	Insoluble	12.1 ± 0.3
Monosaccharides		59.9 ± 3.4
	Glucose	37.8 ± 3.3
	Xylose	17.2 ± 0.0
	Arabinose	2.8 ± 0.0
	Galactose	1.2 ± 0.0
	Mannose	0.6 ± 0.0
	Rhamnose	0.2 ± 0.1
Extractives		20.5 ± 0.5
	Water	17.9 ± 0.3
	Ethanol	2.6 ± 0.2
Proteins		6.6 ± 0.0
Ash		8.3 ± 0.0



**Fig. 3.** Evolution of total lignin composition, acid soluble lignin and acid insoluble lignin (in% dry matter) in the solid fraction as a function of severity factor intensity.

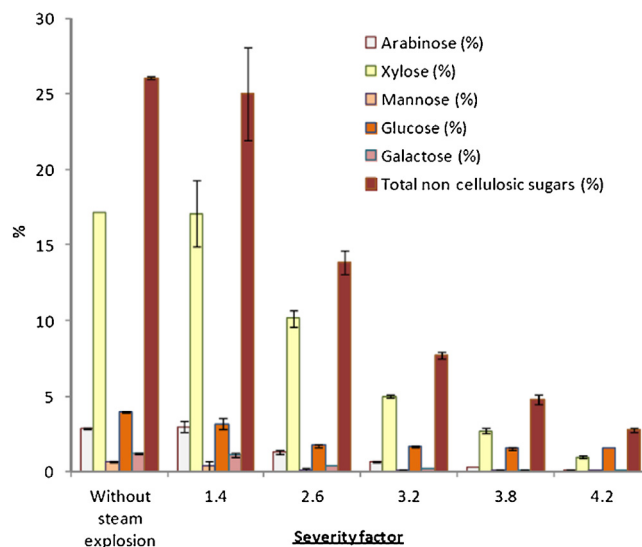
treatment consecutive to the solubilization of water-soluble compounds. For higher intensities ( $S > 2.6$ ), total lignin content stabilizes at approximately 25%. In parallel, ASL in solid fraction decreases with the severity factor increasing. Organic acids released during treatment and thermal environment initiate auto-hydrolysis reactions which affect therefore ASL content (Duquet et al., 2016; Jacquet et al., 2015) (Fig. 3).

Results show also that hemicelluloses content in solid fractions decreases with severity factor increasing (Fig. 4) due to hemicelluloses depolymerization. For highest intensities ( $S = 4.2$ ), hemicelluloses are fully solubilized. Hemicelluloses extraction yield could be estimated by a polynomial correlation (order 2) established between residual hemicelluloses content and severity factor level:  $y = 4, 65x^2 - 56, 82x + 166, 80$  ( $R^2 = 0, 9963$ ).

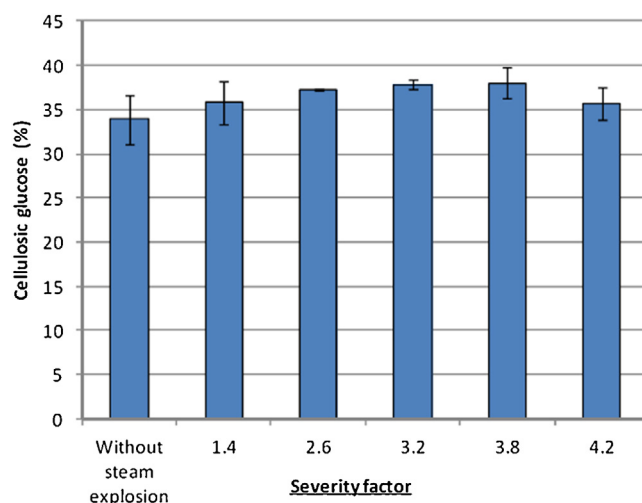
Cellulose content in the solid matter is approximately of 35%, and tends to be relatively stable with the severity factor variation. A slight increase is observed at low intensities, due to the continuous extraction of water-soluble parts (from hemicelluloses). For severity factor higher than  $S = 3.8$ , a slight depreciation is observed, which could indicate the beginning of the cellulose (or rather monomeric glucose) degradation (Jacquet et al., 2011) (Fig. 5).

### 3.3. Combination effects of steam explosion and organosolv process

In order to highlight an eventual benefit of a combinative pretreatment protocol, we investigated the lignin extraction yields



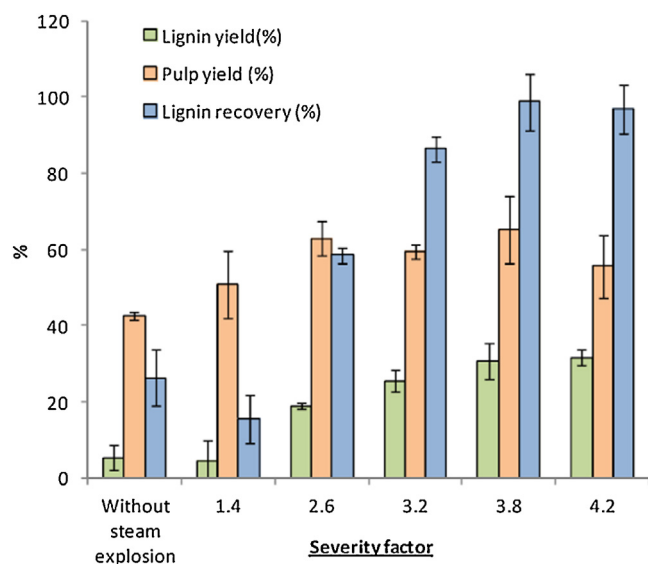
**Fig. 4.** Evolution of total non cellulosic sugars composition (% dry matter) recovered in solid phase as a function of severity factor intensity.



**Fig. 5.** Evolution of cellulose content (% dry matter) in the solid phase as a function of severity factor.

from raw tall fescue submitted to a steam explosion treatment (under various severity conditions ranging from  $S = 1.4$  to  $4.2$ ) combined with an organosolv process. Fig. 6 summarized the “pulp yield” evolution (residual solid part after the both pretreatments), as a function of steam explosion severity conditions, “lignin recovery” (lignin isolated in the liquid phase reported on total lignin content of the raw fescue material), and lignin removal from the raw material, expressed as “lignin extraction yield” (lignin isolated from the liquid phase reported on the lignin content of steam explosion pretreated tall fescue).

Results show that both “lignin extraction yield” and “lignin recovery” are affected by the severity factor  $S$ . Both results increase with steam explosion pretreatment intensity, such as observed in steam explosion treatments followed by a lignin extraction on several other lignocellulosic feedstock including softwood, hardwood, wheat straw and olive tree wood (Cara et al., 2006; Hongzhang and Liying, 2007; Li et al., 2009; Shimizu et al., 1998; Sun et al., 2004). Lignin recovery is from 30% for raw fescue to almost 100% for steam exploded fescue with severity factor values higher than  $S = 3.2$ . These values include potential “pseudolignin” which could be formed by polymerization of polysaccharides degradation prod-

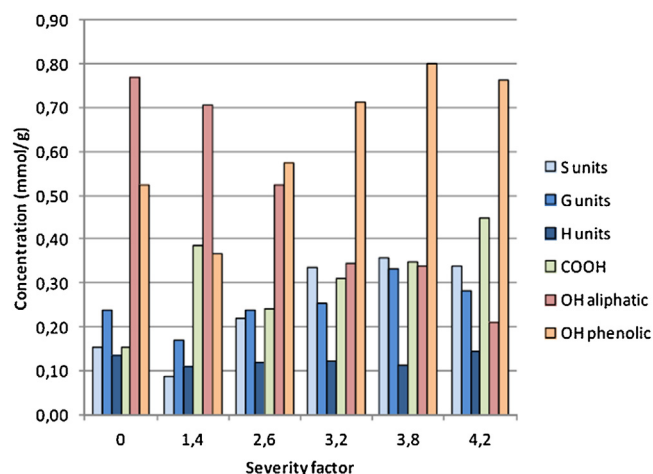


**Fig. 6.** Evolution of lignin yield, pulp yield and lignin recovery (lignin extraction yield/lignin present  $\times 100$ ) obtained after treating tall fescue under organosolv conditions, without or with previous steam explosion (under various severity factors conditions).

ucts (Jakobsons et al., 1995; Li et al., 2009). Pulp yield increases until severity factor of  $S = 2.6$  and tends to remain stable at 60% for more intensive treatments. For low severity factor, soluble molecules present in solid matter are solubilised during acid treatment. This kind of smooth treatment provides more loss of material than high intensity steam explosion treatment. Lignocellulosic deconstruction by steam explosion seems to be more effective with a medium intensity treatment, regarding than a significant increase of lignin yield is not observed for severity factor of  $S > 3.2$ . Lignin recovery increasing could be estimated by the following polynomial equation (order 2) in function of severity factor:  $y = -6,97x^2 + 69,91x - 70,06$  ( $R^2 = 0,9826$ ).

### 3.4. Lignin characterization

Lignin extracted from tall fescue pretreated only with the organosolv (OS) process (OS lignin without steam explosion) has an average molecular weight close to  $1756 \pm 51$  g/mol and a Mw value of  $15329 \pm 644$  g/mol (Table 2). The Mw/Mn ratio (referred as polydispersity index) is  $8.7 \pm 0.1$ . Generally, size exclusion chromatography (SEC) of lignin shows variations depending on lignin structure, pretreatment applied and analytical procedure used for the SEC elution and detection. In this study, Mw and Mw/Mn values are higher than those found in literature (Bonini et al., 2008; Li et al., 2007; Sun et al., 2004; Villaverde et al., 2009) but stay in the range of classic industrial lignin (Majcherczyk and Hüttermann, 1997). The level of Mw/Mn indicates variability in molecular weight of extracted lignin. From a structural point of view, fescue lignin isolated after the organosolv procedure is composed of G, H and S units in proportion of 3:5:3 (Fig. 7, Table 3). COOH units are less abundant and aliphatic hydroxyl groupments are prevalent with a COOH/aliphatic OH proportion of 1:5. High levels of free hydroxyl groups of lignol units are also observed (0.77 mmol/g for aliphatic



**Fig. 7.** Evolution of S units, G units, H units,  $-\text{COOH}$  and aliphatic  $-\text{OH}$  functions concentration with severity factor.

hydroxyl groups and 0.52 mmol/g for phenolic hydroxyl groups). Acetic and formic acids treatment tends to liberate  $\beta\text{-O-4}$  bonds and resulting lignins present a high levels of OH aliphatic groupments (Villaverde et al., 2009). A majority of identified bonds between lignol units are  $\beta\text{-O-4}$  type with spirodienone substructures also detected (Table 3). Presence of ferulates and *p*-coumarates is also observed in switchgrass lignin (Samuel et al., 2011). Fescue purified organosolv lignin does not present a lot of contamination by carbohydrate residues. Two-dimensional NMR does not reveal any characteristic spot of carbohydrate residues and gas chromatography analyses support this observation (less than  $2.4 \pm 0.0\%$  of total carbohydrates). There is no  $\beta\text{-5}$ ,  $\beta\text{-}\beta$ ,  $\beta\text{-1}$ , 5-5, 4-0-5 and dibenzo-1,4-dioxin typical lignin substructures detected in the lignin structure using our methodology.

Combining steam explosion (with several distinct severity factors  $S$ ) and organosolv process allow recovery of lignins with variable molecular weight distributions (Table 2). Result show that both Mw and Mn increase for severity factor 2.6 and 3.2 and decrease when the treatment intensity is higher. Mw/Mn follows the same trend as observed in literature on Norway spruce (Li et al., 2009). Two hypotheses could explain the increase of lignin molecular weight. First, a small part of low lignin molecular weight could be extracted during steam explosion treatment in the liquid phase (decreasing ASL content), resulting of a global increase of lignin molecular weight in solid pre-treated biomass. In parallel, steam explosion process induces simultaneous effects on physico-chemicals properties of lignin. At first, depolymerization influenced by organics acids released during vapocacking step, induces the formation of carbonium ions on  $\text{C}\alpha$ , which break  $\beta\text{-O-4}$  bonds and provide a depolymerization of lignin. Secondly, these ions can react with  $\text{C}_2$  and  $\text{C}_6$  of phenolic ring and generate some polymerization (Bonini et al., 2008; Duque et al., 2016; Jacquet et al., 2015; Li et al., 2009; Sun et al., 2004). These opposite reactions happen simultaneously and depend on physico-chemical and thermal environments that could explain the molecular weight increase observed at low intensities.

**Table 2**

Lignin molecular weight (Mn, Mw and Mw/Mn) obtained after steam explosion-organosolv process reported as a function of the steam explosion severity factor ( $S$ ) evolution.

S.	OS lignin without steam explosion	1.4	2.6	3.2	3.8	4.2
Mn (g/mol)	$1756 \pm 51$	$1896 \pm 53$	$2167 \pm 26$	$2338 \pm 73$	$2147 \pm 30$	$1781 \pm 4$
Mw (g/mol)	$15329 \pm 644$	$20083 \pm 1276$	$23154 \pm 626$	$21334 \pm 356$	$19016 \pm 303$	$14591 \pm 23$
Mw/Mn	$8.7 \pm 0.1$	$10.6 \pm 0.4$	$10.7 \pm 0.2$	$9.1 \pm 0.1$	$8.9 \pm 0.0$	$8.2 \pm 0.0$

**Table 3**  
Identified bonds in lignin (HSQC NMR spectra).

Labels	Assignment	$\delta^{13}\text{C}$ (ppm)	$\delta^1\text{H}$ (ppm)	Sources
1	C <sub>2,6</sub> -H <sub>2,6</sub> Syringyl units etherified (S)	103,7	6,75	(Del Rio et al., 2012a,b; Martinez et al., 2008; Samuel et al., 2011; Villaverde et al., 2009; Wen et al., 2013; Yuan et al., 2011)
2	C <sub>2</sub> -H <sub>2</sub> in guaiacyl units (G)	111,0	6,79	(Del Rio et al., 2012a,b; Villaverde et al., 2009; Wen et al., 2013; Yuan et al., 2011)
3	C <sub>5</sub> -H <sub>5</sub> in guaiacyl units (G)	115,2	6,80	(Samuel et al., 2011; Villaverde et al., 2009)
4	C <sub>5</sub> -H <sub>5</sub> and C <sub>6</sub> -H <sub>6</sub> in guaiacyl units (G)	119,1	6,90	(Del Rio et al., 2012a,b; Martinez et al., 2008; Samuel et al., 2011; Villaverde et al., 2009; Wen et al., 2013; Yuan et al., 2011)
5	C <sub>2,6</sub> -H <sub>2,6</sub> in <i>p</i> -hydroxyphenyl units (H)	127,8	7,20	(Del Rio et al., 2012a; Martinez et al., 2008; Samuel et al., 2011; Wen et al., 2013; Yuan et al., 2011)
6	C <sub>2,6</sub> -H <sub>2,6</sub> in <i>p</i> -hydroxyphenyl units (H)	129,0	7,26	(Del Rio et al., 2012a,b; Samuel et al., 2011; Wen et al., 2013; Yuan et al., 2011)
7	C <sub>2,6</sub> -H <sub>2,6</sub> in <i>p</i> -coumarate	130,2	7,49	(Del Rio et al., 2012a,b; Samuel et al., 2011; Villaverde et al., 2009; Yuan et al., 2011)
8	C <sub>2</sub> -H <sub>2</sub> in Ferulate	111,2	7,34	(Del Rio et al., 2012a,b)
9	C $\alpha$ -H $\alpha$ in ferulate and <i>p</i> -coumarate	144,6	7,64	(Del Rio et al., 2012a,b; Samuel et al., 2011)
A	OMe	55,7	3,73	(Del Rio et al., 2012a,b; Samuel et al., 2011; Wen et al., 2013; Yuan et al., 2011)
B	C $\gamma$ H $\gamma$ in $\beta$ -O-4 substructures	60,1	3,75	(Del Rio et al., 2012a,b; Samuel et al., 2011; Wen et al., 2013; Yuan et al., 2011)
C	C $\gamma$ H $\gamma$ in $\gamma$ -acylated $\beta$ -O-4 substructures	63,0	4,38	(Balakshin et al., 2011; Capanema et al., 2004; Del Rio et al., 2012a,b; Villaverde et al., 2009; Wen et al., 2013; Yuan et al., 2011)
D	C $\alpha$ -H $\alpha$ in $\beta$ -O-4' substructures	71,3	4,91	(Capanema et al., 2004; Del Rio et al., 2012a,b; Samuel et al., 2011; Wen et al., 2013; Yuan et al., 2011)
E	C $\beta'$ -H $\beta'$ in spirodienone substructures	79,6	4,17	(Balakshin et al., 2011; Yuan et al., 2011)

For higher intensities  $S > 3.2$ , molecular weight decreasing results of the increase of the lignin depolymerisation during steam explosion step.

Combination of steam explosion with acetic and formic acid process has also effects  $\beta$ -O-4 bonds that tend to disappear when severity factor is higher than 2.6. Precisely, 2D HSQC spectra show a decreasing of signal for C $\alpha$  and C $\gamma$  in  $\beta$ -O-4 bonds and for C $\gamma$  acetylated with application of steam explosion intensities compared to organosolv fescue lignin as observed in Li et al. on aspen wood. Acetylated C $\alpha$  is however not detected. Spirodienone substructures fade out also at higher steam explosion treatment intensities. As observed in literature on steam exploded lignin from different woods (Li et al., 2009),  $^{31}\text{P}$  NMR spectra illustrate an increase, with severity factor intensification, of phenolic hydroxyls and carbonyl compounds induced by lignin degradation (Fig. 7). At the opposite, level of aliphatic hydroxyl groupments tends to decrease with severity factor that could reduce lignin reactivity. Acetylation intensification of aliphatic hydroxyl groupments (revealed by  $^{31}\text{P}$  NMR) during organosolv extraction could explain this decreasing. This fact is already observed on organosolv lignin where aliphatic hydroxyl groupments are more affected by acetylation during extraction while phenolic hydroxyl groupments are not (Delmas, 2011). Following these observations, aliphatic hydroxyl functional groupments are indeed important for the establishment of a probant antioxidant activity as aromatic hydroxyl and are implied in the resonance stabilization of lignin when reacting with free radicals. However, the increase of phenolic hydroxyl groupments can significantly affect the chemical reactivity of the material that could promote the synthesis of new chemical active sites on lignin by various process such as nitration, amination, alkylation/dealkylation, carboxylation and halogenation, etc. (Laurichesse and Avérous, 2014).

G and S units occurrence and proportion seem to be influenced by depolymerization process as viewed on steam-exploded lignin in Li and Shimizu (Li et al., 2009; Shimizu et al., 1998). Ferulates and *p*-coumarates concentration decreases when the steam explosion conditions are harsh (Table 3). Steam exploded lignins present a

great level of purity with a few carbohydrate residues as monitored by 2D-NMR. As quantified using gas chromatography, carbohydrates contamination of  $2.0 \pm 0.1\%$  for severity factor of 1.4 and  $< 1.0\%$  for the more intensive treatment was detected.

#### 4. Conclusion

Fescue analysis show that dry matter contains 60% of potential sugar, distributed mainly between cellulose and hemicelluloses fractions. Steam explosion, combined with organic acids treatment increases significantly lignin yields extraction at higher level than organosolv pretreatment used alone, results show also that lignin extraction tends to be more effective with medium intensities steam explosion levels ( $S = 3.2$ – $3.8$ ). Moreover, Study highlights that isolated lignins obtained purity level sought for high added value applications in biorefinery. Depending of treatment severity, steam explosion induces various chemical and physico-chemical modifications on lignin such as molecular mass and intramolecular chemical bonds. Phenolic OH and  $-\text{COOH}$  groups tend to increase while aliphatic OH decrease with the increase of severity factor intensity. Lignin composition is also influenced by the harshness of the steam explosion treatment, especially G/H/S units ratio and the presence of ferulates and *p*-coumarates fractions.

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.indcrop.2017.01.015>.

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