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## Proceedings





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## STRUCTURAL CHARACTERIZATION OF THE MILLED WOOD LIGNIN ISOLATED FROM BREWER'S SPENT GRAIN

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### ABSTRACT

Brewer's spent grain (BSG) is the major by-product generated by the brewing industry. BSG is rich in carbohydrates, lignin, proteins and lipids, and has a high potential as source of food, energy and chemicals. In this work, the composition and structural characteristics of the lignin from BSG have been studied in detail by Py-GC/MS and 2D-NMR. The data demonstrated that this lignin is a *p*-hydroxyphenyl-guaiacyl-syringyl (H-G-S) lignin, with a predominance of G units (S/G ratio of 0.4). 2D-NMR indicated that the main substructures present include  $\beta$ -O-4' alkyl-aryl ethers followed by small amounts of phenylcoumarans and resinols.

### I. INTRODUCTION

Brewer's spent grain (BSG) is the solid residue obtained from barley (*Hordeum vulgare* L.) after mashing and filtration from the brewing process. BSG basically consists of the husk-pericarp-seed coat layers that covered the original barley grain [1]. BSG represents up to 30% (w/w) of the starting malted grain, which makes this a readily available, high volume and low cost by-product within the brewing industry, and a potentially valuable resource for industrial exploitation. BSG is a lignocellulosic material containing cellulose (17-25%), non-cellulosic carbohydrates (25-35%), protein (15-24%) and lignin (8-28%), with lower amounts of lipids (10%) [1,2]. For an appropriate valorization of BSG as a source for added-value products, the complete characterization of the different components present is of high interest. Previous studies have mostly dealt with the composition of carbohydrates, proteins, lipids and *p*-hydroxycinnamic acids [2,3]. In comparison, studies concerning the composition and structure of lignin in BSG have been relatively scarce and mostly limited to its interactions with gastrointestinal microbiota [4]. In this paper, an in-depth and complete characterization of the lignin polymer of BSG has been performed. For this, a 'milled-wood' lignin (MWL) preparation was isolated according to traditional lignin isolation procedures, which was subsequently analyzed by Py-GC/MS and 2D-NMR.

### II. EXPERIMENTAL

#### *Samples*

BSG was obtained from Adnams brewery (Southwold, UK) and was kindly provided by Prof. Craig B. Faulds (INRA, Marseille). Klason lignin content was estimated as the residue after sulphuric acid hydrolysis of the pre-extracted material according to the TAPPI method T222 om-8. The Klason lignin content was then corrected for proteins, determined from the N content and using a 6.25 factor, and ash, estimated as the residue after 6 h of heating at 575 °C. The acid-soluble lignin was determined, after the insoluble lignin was filtered off, by UV-spectroscopic determination at 205 nm wavelength using 110 L cm<sup>-1</sup> g<sup>-1</sup> as the extinction coefficient.

#### *Milled-wood lignin' isolation*

The lignins were obtained according to the classical procedure, and the detailed protocol has been explained somewhere else [5,6]. The final yields were 10% of the original Klason lignin content.

#### *Analytical pyrolysis*

Pyrolysis of MWL (approximately 100 µg) was performed with a 3030 micro-furnace pyrolyzer (Frontier Laboratories Ltd.) connected to an Agilent 7820A GC using a DB-1701 fused-silica capillary column (60 m x 0.25 mm i.d., 0.25 µm film thickness) and an Agilent 5975 mass selective detector (EI at 70 eV). The pyrolysis was performed at 500 °C. The oven temperature was programmed from 45 °C (4 min) to 280 °C (10 min) at 4 °C min<sup>-1</sup>. Helium was the carrier gas (1 mL min<sup>-1</sup>). Peak molar areas were calculated for the lignin-degradation products, the summed areas were normalized and expressed as percentages.

#### *NMR spectroscopy*

2D-NMR spectra were recorded at 25 °C on a Bruker AVANCE III 500 MHz instrument, equipped with a cryoprobe. MWL (40 mg) was dissolved in 0.75 mL of dimethylsulfoxide (DMSO)-*d*<sub>6</sub>. The central solvent peak

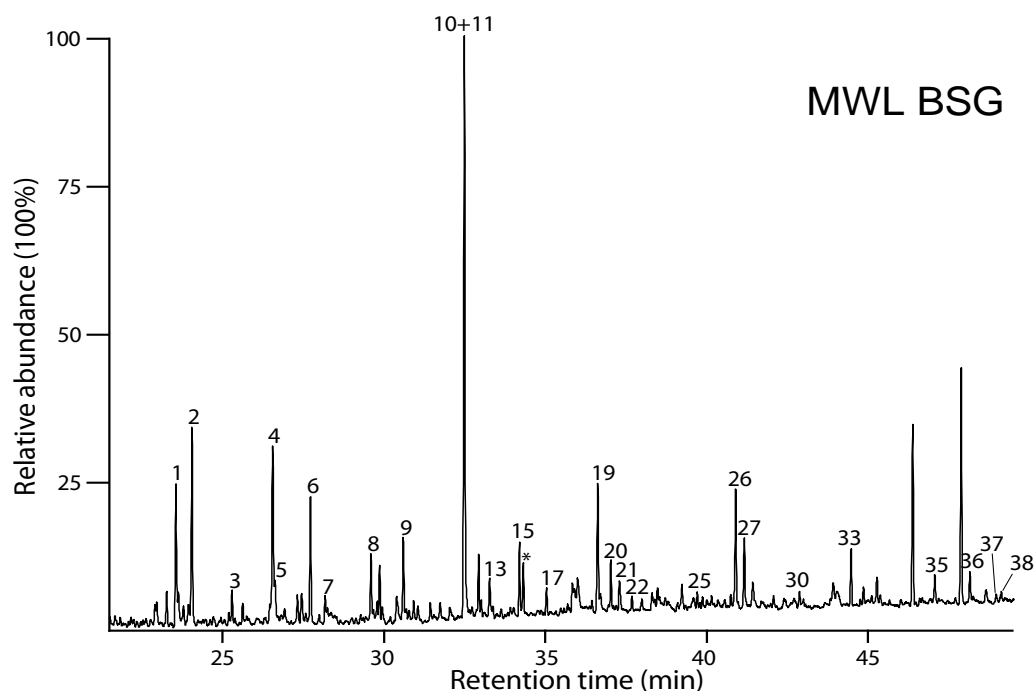
was used as internal reference (DMSO  $\delta_C/\delta_H$  39.5/2.49). The HSQC (heteronuclear single quantum coherence) experiment used Bruker's "hsqcetgpsisp2.2" pulse program (adiabatic-pulsed version) with spectral widths of 5000 Hz and 20,843 Hz for the  $^1\text{H}$ - and  $^{13}\text{C}$  dimensions. The number of transients was 64, and 256 time increments were always recorded in the  $^{13}\text{C}$  dimension. The  $J_{\text{CH}}$  used was 145 Hz. Processing used typical matched Gaussian apodization in the  $^1\text{H}$  dimension and squared cosine-bell apodization in the  $^{13}\text{C}$  dimension. Prior to Fourier transformation, the data matrices were zero-filled up to 1024 points in the  $^{13}\text{C}$ -dimension. 2D-NMR cross-signals were assigned by literature comparison [5-7]. A semiquantitative analysis of the HSQC correlation peaks was performed using Bruker's Topspin 3.1 processing software. Relative abundances of inter-unit linkages were estimated from  $\text{C}_\alpha\text{-H}_\alpha$  correlations, and the relative abundance of side-chains involved in different substructures and terminal structures were calculated. In the aromatic/unsaturated region,  $\text{C}_2\text{-H}_2$  from G and  $\text{C}_{2,6}\text{-H}_{2,6}$  from S lignin units were used to estimate their abundances.

### III. RESULTS AND DISCUSSION

The Klason lignin content of the BSG was relatively low (8.8%) compared to previous published data for the same sample (16-20.1%). The main reason for this discrepancy is that previous works did not consider the high amounts of proteins and ashes when quantifying the Klason lignin content, which was corrected here. In this work, we have thoroughly studied the lignin composition and structure of BSG. For this purpose, a 'milled-wood' lignin (MWL) preparation was isolated and was then analyzed by Py-GC/MS and 2D-NMR.

#### Py-GC/MS

The pyrogram of the MWL from BSG is shown in **Figure 1**. Pyrolysis released phenolic compounds that are derived from *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) lignin units. The most predominant phenolic compounds were phenol (1), guaiacol (2), 4-methylphenol (4), 4-methylguaiacol (6), 4-vinylphenol (10), 4-vinylguaiacol (11), syringol (15), *trans*-isoeugenol (19), 4-methylsyringol (20), 4-vinylsyringol (26), guaiacylacetone (27) and *trans*-4-propenylsyringol (33). The high levels of phenol, 4-methylphenol and 4-vinylphenol released, together with the presence of indol, indicates a major contribution from proteins. In addition, the high amounts of 4-vinylphenol released upon pyrolysis, as also occurs in other grasses, also point to the presence of *p*-coumarates esters, which decarboxylates under pyrolytic conditions [5-9]. Similarly, 4-vinylguaiacol (11), which is present in high abundance, also arises from ferulates after decarboxylation upon pyrolysis. A rough estimation of the S/G ratio (by using the molar areas of all G- and S-derived compounds, except 4-vinylguaiacol, that also arises from ferulates, and its respective 4-vinylsyringol), indicate a S/G ratio of 0.4. The occurrence of *p*-hydroxycinnamates in BSG was assessed by pyrolysis in the presence of TMAH [5-9]. Previous studies have indicated that *p*-coumarates in grasses are esterified to the lignin side-chains, and more specifically acylates the  $\gamma\text{-OH}$  of the lignin side-chain [5,6,10,11].

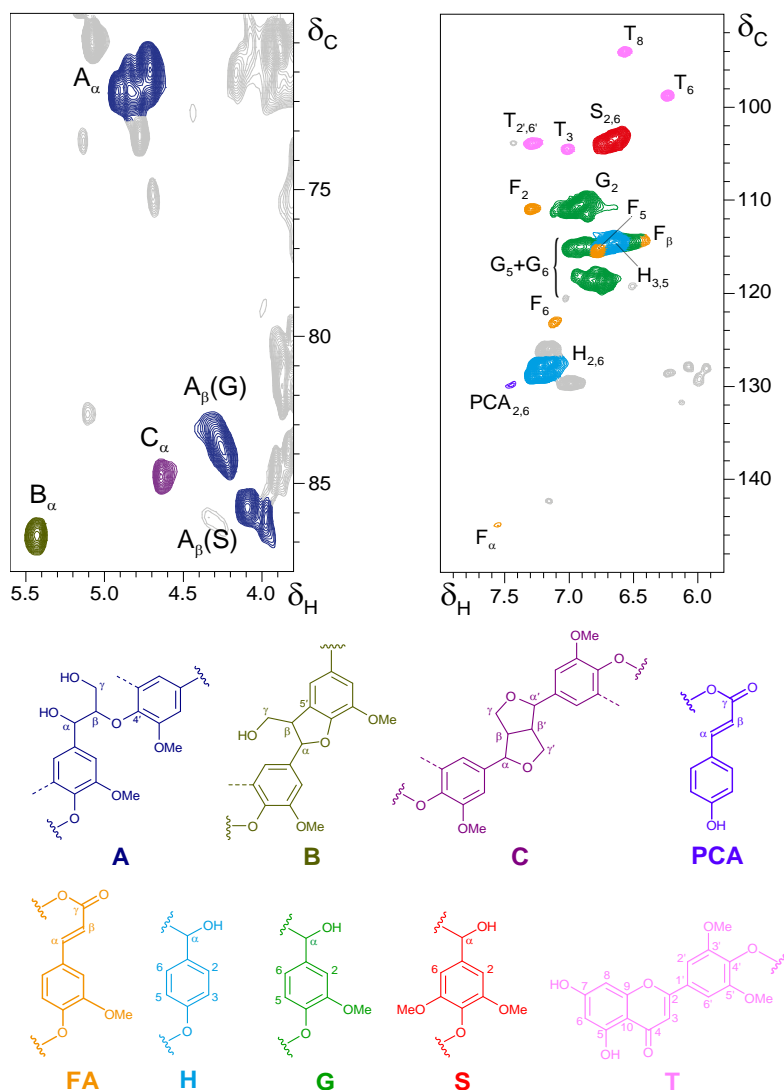


**Figure 1.** Py-GC/MS of the MWL isolated from BSG.

## 2D-NMR

The MWL from BSG was also analyzed by 2D-NMR. The side-chain ( $\delta_C/\delta_H$  69–88/3.8–5.6) and the aromatic ( $\delta_C/\delta_H$  90–150/5.8–8.0) regions of the spectrum are shown in **Figure 2**. The main substructures present are also depicted in **Figure 2**. The spectrum shows prominent signals corresponding to  $\beta$ -O-4' aryl-ether linkages (**A**), together with smaller signals for phenylcoumarans (**B**), resinols (**C**) and dibenzodioxocins (**D**). The main cross-signals in the aromatic region of the HSQC spectra corresponded to the aromatic rings of the H, G and S lignin units, and the *p*-hydroxycinnamates ferulates and *p*-coumarates. Strong signals corresponding to C<sub>3,5</sub>-H<sub>3,5</sub> and C<sub>2,6</sub>-H<sub>2,6</sub> correlations in "H-lignin" units were observed, confirming that the high abundance of 'H-units' observed upon pyrolysis was also due to the presence of proteins. Interestingly, in this region of the HSQC spectra, it was also possible to detect two characteristic signals at  $\delta_C/\delta_H$  94.1/6.56 and 98.8/6.20 corresponding to the C<sub>8</sub>-H<sub>8</sub> and C<sub>6</sub>-H<sub>6</sub> correlations of tricrin (**T**), a flavone that is apparently incorporated into the lignins in some grasses [6], and that also occur in other monocotyledons, as in the lignin of coconut coir [7].

The relative abundances of the main lignin inter-unit linkages, the molar abundances of the different lignin units (H, G and S), and *p*-coumarates, ferulates and tricrin, are shown in **Table 1**. The main substructure present in the lignin of BSG was the  $\beta$ -O-4' aryl ether, that accounts for 91% of all inter-unit linkages, followed by smaller amounts of phenylcoumaran that involved 6.8% of all linkages and resinols with 2.2%. The lignin S/G ratio determined upon NMR (0.4) was similar to that obtained upon Py-GC/MS.



**Figure 2.** Side-chain and aromatic/unsaturated regions in the HSQC spectrum of the MWL from BSG. Main structures present: (**A**)  $\beta$ -O-4' alkyl-aryl ethers; (**B**) phenylcoumarans; (**C**) resinols; (**PCA**) *p*-coumarates; (**FA**) ferulates; (**H**) *p*-hydroxyphenyl units; (**G**) guaiacyl units; (**S**) syringyl units; (**T**) tricrin.

**Table 1.** Structural Characteristics (Lignin Inter-Unit Linkages, Aromatic Units, *p*-Coumarates and Ferulates Content) of the MWL Isolated from Brewer's Spent Grain

	Abundance
Lignin inter-unit linkages (%)	
$\beta$ -O-4' aryl ethers ( <b>A</b> )	72
C $\alpha$ -oxidized $\beta$ -O-4' aryl ethers ( <b>Aox</b> )	5
Phenylcoumarans ( <b>B</b> )	12
Resinols ( <b>C</b> )	6
Dibenzodioxocins ( <b>D</b> )	5
Lignin aromatic units	
H (%)	31
G (%)	51
S (%)	18
S/G ratio	0.35
<i>p</i> -Hydroxycinnamates	
<i>p</i> -Coumarates (%)	2
Ferulates (%)	7
<i>p</i> -Coumarates/Ferulates ratio	0.28
Tricin	6

#### IV. CONCLUSIONS

Py-GC/MS and 2D-NMR analysis of the MWL isolated from BSG indicated that it is a H:G:S lignin with a S/G ratio of 0.4, and with some amounts of associated *p*-coumarates and ferulates. The main lignin inter-unit linkages present was the  $\beta$ -O-4' aryl ether, followed by smaller amounts of phenylcoumaran and resinols. The flavone triclin was incorporated into this lignin, as also occurs in other grasses.

#### V. ACKNOWLEDGEMENT

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#### VI. REFERENCES

- [1] Mussatto, S.I.; Dragone, G.; Roberto, I.C. Brewers' spent grain: generation, characteristics and potential applications. *J. Cer. Sci.* **2006**, *43*, 1–14.
- [2] Robertson, J.A.; l'Anson, K.J.A.; Treimo, J.; Faulds, C.B.; Brocklehurst, T.F.; Eijssink, V.G.H.; Waldron, K.W. Profiling brewers' spent grain for composition and microbial ecology at the site of production. *LWT-Food Sci. Technol.* **2010**, *43*, 890–896.
- [3] del Río, J. C.; Prinsen, P.; Gutiérrez, A. Chemical composition of lipids in brewer's spent grain: A promising source of valuable phytochemicals. *J. Cereal Sci.* **2013**, *58*, 248–254.
- [4] Niemi, P.; Aura, A.-M.; Maukonen, J.; Smeds, A.I.; Mattila, I.; Niemelä, K.; Tamminem, T.; Faulds, C.B.; Buchert, J.; Potanen, K. Interactions of a lignin-rich fraction from brewer's spent grain with gut microbiota in vitro. *J. Agric. Food Chem.* **2013**, *61*, 6754–6762.
- [5] del Río, J.C.; Prinsen, P.; Rencoret, J.; Nieto, L.; Jiménez-Barbero, J.; Ralph, J.; Martínez, A.T.; Gutiérrez, A. Structural characterization of the lignin in the cortex and pith of elephant grass (*Pennisetum purpureum*) stems. *J. Agric. Food Chem.* **2012**, *60*, 3619–3634.
- [6] del Río, J.C.; Rencoret, J.; Prinsen, P.; Martínez, A.T.; Ralph, J.; Gutiérrez A. Structural characterization of wheat straw lignin as revealed by analytical pyrolysis, 2D-NMR, and reductive cleavage methods. *J. Agric. Food Chem.* **2012**, *60*, 5922–5935.
- [7] Rencoret, J.; Ralph, J.; Marques, G.; Gutiérrez, A.; Martínez, A.T.; del Río, J.C. Structural characterization of lignin isolated from coconut (*Cocos nucifera*) coir fibers. *J. Agric. Food Chem.* **2013**, *61*, 2434–2445.
- [8] del Río, J.C.; Gutiérrez, A.; Rodríguez, I.M.; Ibarra, D.; Martínez A.T. Composition of non-woody plant lignins and cinnamic acids by Py-GC/MS, Py/TMAH and FT-IR. *J. Anal. Appl. Pyrol.* **2007**, *79*, 39–46.
- [9] del Río, J.C.; Martín, F.; González-Vila, F.J. Thermally assisted hydrolysis and alkylation as a novel pyrolytic approach for the structural characterization of natural biopolymers and geomacromolecules. *Trends Anal. Chem.* **1996**, *15*, 70–79.
- [10] Ralph, J.; Hatfield, R.D.; Quideau, S.; Helm, R.F.; Grabber, J.H.; Jung, H.-J.G. Pathway of *p*-coumaric acid incorporation into maize lignin as revealed by NMR. *J. Am. Chem. Soc.* **1994**, *116*, 9448–9456.
- [11] Ralph, J. Hydroxycinnamates in lignification. *Phytochem. Rev.* **2010**, *9*, 65–83.