Article

Fluorescence Labelling of Technical Lignin for the Study of Phenolic Groups Distribution as a Function of the Molecular Weight

Anika Salanti, Marco Orlandi, and Luca Zoia

ACS Sustainable Chem. Eng., **Just Accepted Manuscript** • DOI: 10.1021/acssuschemeng.0c01571 • Publication Date (Web): 11 May 2020

Fluorescence Labelling of Technical Lignin for the Study of Phenolic Groups Distribution as a Function of the Molecular Weight

Anika Salanti[†], Marco Orlandi[†], Luca Zoia[†]*

[†]Department of Earth and Environmental Sciences, University of Milano-Bicocca, Piazza della Scienza, 1 Milan, I-20126 Italy

[*]Corresponding author: telephone +39 02 6448 2919; e-mail marco.orlandi@unimib.it

ABSTRACT

A novel analytical approach based on fluorescence labelling was developed in the effort to increase the understanding of phenolic group distribution in technical lignins. Selective derivatization with a fluorophore (dansyl chloride) of lignin phenolic functionalities was quantitatively achieved under mild reaction conditions. Reference acetylated lignin and labelled lignin were analyzed by Gel Permeation Chromatography (GPC) coupled to a UV-Vis detector (set at 280 nm) and a florescence detector (λ excitation: 390 nm, λ emission: 550 nm) to discern the dansyl-linked phenols response from the lignin aromatic skeleton input. After data elaboration, valuable information about phenolic group distribution as a function of molecular weight for different technical lignin was gathered. This novel analytical approach is applied to model lignin polymer thermal protection properties, a useful parameter in lignin valorization strategies.

KEYWORD

Technical lignin; phenol; dansyl; fluorescence; molecular weight.

INTRODUCTION

Lignin is a three-dimensional, heterogeneous and irregular polyphenolic macromolecules and the second most abundant natural polymer after cellulose. Along with hemicelluloses, these biopolymers constitute the so-called lignocellulosic materials. Lignin is constituted by three phenolic monomers, named p-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units when linked into the lignin polymer through ether and carbon-carbon bonds.[1] The structure of lignin, as well as the amount of the three monomeric units, depends on the botanical origin and the type of lignocellulosic biomass. Approximately 50 million tons of technical lignins are annually produced from spent pulping liquors, mainly from the sulphite and kraft processes.[2] During the pulp and paper production, intermolecular linkages are broken and modified to separate lignin from the lignocellulosic composite and the already complicated lignin structure is strongly affected by these treatments.[3] The heterogeneity of the resulting technical lignin is the major drawback for its valuable valorization.[4] Several papers were focused on different fractionation approaches to extract more homogenous lignin preparation in terms of molecular weight and functional groups distribution.[5] It was demonstrated that sequential ultrafiltration of kraft lignin is able to fractionate and elucidate molar mass-dependent changes in lignin structure and characteristics, revealing that the smaller fractions are also the richest in phenolic groups.[6–8] Selective solvent extraction, the oldest fractionation techniques described, takes advantage of the different solubility of lignin in solvents with varying polarity.[9] In general, the lower the solvent polarity, the lower the average molecular weight of the fraction and the largest the phenolic content.[10–12] Fractionation by successive precipitation, obtained either by a stepwise reduction of the pH value of black liquor or especially by the addition of a non-solvent to an organic lignin solution, generally results in high lignin yield and highly monodispersed fractions.[13–15]

Despite these efforts, a strong contribution in the field of the analytical chemistry of lignin is still necessary to deeply understand and completely control a lignin up-grading. As reported by Potthast and co-workers, "in general, from an analytical point of view, the understanding of technical lignins is set back by the common analysis approach - putting together fragments with known structural features and perhaps some newly identified motifs and complementing this by analyzing many functional groups. However, we have not yet arrived at a stage where we can state that we comprehend the whole picture of this fascinating molecule".[7] The presented study is focused on the possibility to obtain a precise knowledge about the distribution of phenolic groups in technical lignins as a function of their molecular weight, avoiding the need of a preliminary lignin fractionation process. Fractionation procedures are indeed expensive in terms of time, resources and effort whereas, according to the method herein described, a GPC analysis performed on the lignin sample as a whole might be enough to select a specific fraction of interest. Otherwise, the generally proposed workflow involving fractionation followed by fractions analysis might be replaced by the inverse, plainer approach analysis-fractionation. In particular, the proposed analytical method is based on the selective fluorescence labelling of lignin phenolic moieties with dansyl chloride. The phenols were chosen as main representative and versatile functional group on account of their properties and easiness of conversion to other reactive species. The selectivity and yield of the labelling reaction were determined by quantitative phosphorylation of phenols followed by ^{31}P-NMR.[16] The fluorescence-labelled lignin, along with the acetylated reference, was then analyzed by Gel Permeation Chromatography (GPC), performed on an instrument connected to a UV-Vis and a fluorescence detector. The comparison between the acetylated and dansylated GPC profiles, properly evaluated and computed, returned helpful information about phenols concentration over different molecular weight ranges.

EXPERIMENTAL

Reagents and Materials. All solvents and reagents were purchased from Sigma-Aldrich Italia and used as received. Technical lignins from the Kraft process (softwood kraft, SWK; hardwood kraft, HWK), soda pulping (soda grass, SG) and steam explosion (wheat straw, WS_{SE}) were kindly provided by local factories. The WS_{SE} was further purified by an acidic-basic treatment according to the optimized conditions described by Zoia et al.[17]

Lignin Acetylation. Lignin samples (ca. 50 mg) were acetylated using a 1:1 pyridine/acetic anhydride mixtures (2 mL, 40°C, 24 h) and recovered by stripping with EtOH, toluene and chloroform before being subjected to GPC analysis.

Lignin Dansylation. In a typical experimental procedure, 100 mg of lignin and 5 mg of tetrabutylammonium bromide (TBAB) were dissolved in 20 mL of carbonate buffer solution (pH 10, 0.1 M) and heated to 50°C in an oil bath. A certain amount of dansyl chloride (20% molar excess to the total phenolic content as calculated from ^{31}P-NMR) was then solubilized in acetonitrile (10 mL) and added to the lignin solution. The mixture was reacted for 90 minutes at 50°C under vigorous stirring. At the end of the reaction, dansylated lignin was recovered by centrifugation (4000 rpm, 10 min, 2 cycles washing with acidic water) after regeneration in cold acidic water (HCl 0.1 M). For the purpose of this work, the yield of the dansylation reaction was evaluated by ^{31}P-NMR as % of reacted phenols. (Full spectra are provided as Supporting Information).

Model Compounds Synthesis. Dimeric model compounds of vanillyl alcohol (MW 154.2 g/mol) and isoeugenol (MW 164.2 g/mol) were obtained via enzymatic oxidative coupling.[18] 500 mg of monomer were dissolved in a 3:1 phosphate buffer/dioxane solution (phosphate buffer 0.05 M, pH 6.0). Afterwards, 1 mg of type VI-A horseradish peroxidase (activity: 950-2000 units/mg solid using ABTS) was added. The amount of oxidant (hydrogen peroxide, H_2O_2, 30% w/v in water) was calculated according to a 1:0.5 model compound/H_2O_2 ratio,

prepared as a 3% solution in water and added to the reaction mixture under gentle stirring in 5 aliquots, every 5 min, over an average period of 30 minutes. During the addition of the oxidant, the reaction was monitored by thin layer chromatography (TLC, eluent 7:3 hexane/ethyl acetate). The products were extracted into ethyl acetate, washed twice with slightly acidic water, dried over anhydrous sodium sulfate and vacuum dried. The crude was purified on silica flash column (silica gel 60, 230-400 mesh ASTM, Merck) eluting with hexane/ethyl acetate 6:4. For the purpose of this work, both the dimer and the residual monomer were recovered and used as a representative simple mixture to validate the chromatographic method developed. The linkage distributions of the oxidation products were detected by ^{1}H-NMR and ^{13}C-NMR. The average phenolic content of the mixtures was assessed by quantitative ^{31}P-NMR analysis. (Spectra are provided as Supporting Information).

Model Compounds Dansylation. The dansylation reaction on model compounds was accomplished using the same conditions described above for lignin except that, after the addition of the reaction mixture to cold acidic water, the pH was adjusted to 3 to optimize the products precipitation and then the dansylated model compounds were extracted in ethyl acetate, anhydrified over sodium sulphate and rotary evaporated. The conservation of the intact dansyl units was verified by ^{1}H-NMR and ^{13}C-NMR. The yield of the reaction, in terms of reacted phenolic functionality, was assessed by ^{31}P NMR. (Spectra are provided as Supporting Information).

^{31}P NMR Analyses. Quantitative ^{31}P-NMR analyses were performed according to a well-established procedure[16] using 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane as phosphorus derivatizing agent. The ^{31}P-NMR data reported in this paper are the average of three experiments. The maximum standard deviation was 0.02 mmol/g, while the maximum standard error was 0.01 mmol/g.

GPC-UV-Fluorescence Analysis. GPC analyses were performed on an Agilent 1260 Infinity liquid chromatography system, equipped with an autosampler (Agilent 1260 Vialsampler, injection volume 25 μL) and connected in series to an Agilent 1260 DA VL detector (set at 280 nm) and an Agilent 1260 FL detector (λ excitation: 390 nm, λ emission: 550 nm for all samples). The GP-column system was composed as follows (according to the solvent flow direction): Agilent PLgel 5 μm, 500 Å, Agilent PLgel 5 μm, 10 00 Å, and Agilent PLgel 5 μm, 10000 Å. Samples were dissolved in tetrahydrofuran (THF, accurately prepared at the concentration of 1 mg/mL starting from more concentrated stock solutions) and analyzed using THF as eluent (Fluka 99.8%) at a flow rate of 1 mL/min. PL Polymer Standards of Polystyrene from Polymer Laboratories were used for calibration. The evaluation of the number-average molecular weight (M_n) and the weight-average molecular weight (M_w) of the acetylated lignin samples was performed. The peak molecular weight M_p is defined as the molecular weight of the species with maximum absorbance. Moreover, the ratio $I = M_w/M_n$, defined as PolyDispersity Index was also calculated. The M_n, M_w, and M_p values reported are the average of three analyses (standard error M_w: 1000 g/mol; M_n, M_p: 100 g/mol). The phenol content, calculated from GPC-FL data, reported in this paper are the average of three experiments. The maximum standard deviation was 0.2 mmol/g, while the maximum standard error was 0.1 mmol/g.

RESULTS AND DISCUSSION

Dansylation reaction

As reported in experimental part, lignin samples were labelled with dansyl chloride (Figure 1) and then characterized by ^{31}P-NMR in order to study the yield and the selectivity of the reaction (full spectra provided as Supporting Information).

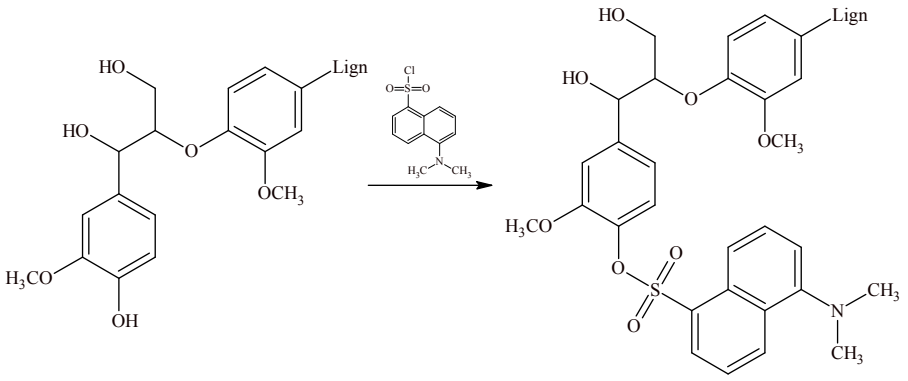


Figure 1. Dansylation reaction for a typical β-O-4 structure of guaiacylic nature.

The results after the dansylation reaction on the SWK, HWK, SG, and WS_{SE} lignins are reported in Table 1. The reaction sites taken in consideration were alcohols, carboxylic acids and phenols. Concerning HW and SW kraft lignin, the presence of sulfur had also to be considered: kraft lignin samples could contain around 2-3% of S, roughly 10% of which is in form of thiols.[14] Anyway, the formation of thiosulfonates lignin derivatives did not seem feasible according to the applied reaction conditions. For the aim of this work, firstly a mass ratio for each lignin was calculated as the ratio between the starting weight and the final weight of lignin after the dansylation reaction (Table 1, first line). The experimental mass ratios were in line with the theoretical values calculated by adding 233.3 g/mol (the mass of dansyl unit) for each phenolic functionality, supposing that phenols were the only reactive group and assuming a 100% conversion (0.51 *vs* 0.49 for SWK, 0.60 *vs* 0.59 for HWK, 0.55 *vs* 0.53 for SG and 0.67 *vs* 0.69 for WS_{SE}).

The mass ratios were then used to assess the reliability and selectivity of the dansylation reaction. The comparison between the experimental quantification in mmol/g, obtained by the ^{31}P-NMR of aliphatic alcohols and carboxylic acids (second and fourth line, Table 1) before (corrected using the mass ratios and reported in bracket) and after dansylation, resulted in a satisfying match between the two values. The dansylation reaction occurred mainly at the

phenolic sites and the total amount of phenols (sixth line, Table 1) detected after the derivation approached zero for every samples. The percentages of the conversion were used to evaluate of the actual yield of the dansylation reaction (always > 90%). In conclusion, the described labelling reaction was sufficiently selective and complete, and was considered a good candidate for the study of lignin phenolic groups distribution.[19]

	SWK	**SWK Dans**	**HWK**	**HWK Dans**	**SG**	**SG Dans**	**WS_{SE}**	**WS_{SE} Dans**
Mass Ratio	0.51		0.60		0.55		0.69	
Aliph -OH (^{31}P NMR, mmol/g)	2.02 (1.03)	0.82	1.12 (0.67)	0.56	1.84 (1.01)	0.77	2.02 (1.39)	1.10
Aliph-OH conversion (%)	20		17		24		21	
COOH (^{31}P NMR, mmol/g)	0.52 (0.27)	0.28	0.38 (0.23)	0.21	1.04 (0.57)	0.34	0.68 (0.47)	0.38
COOH Conversion (%)	-5		8		40		19	
PhOH (^{31}P NMR, mmol/g)	4.39 (2.24)	0.15	2.93 (1.76)	0.15	3.71 (2.04)	0.09	2.09 (1.44)	0.07
PhOH Conversion (%)	93		91		96		95	

Table 1. Mass Ratio (starting weight/final weight of lignin after the dansylation reaction), experimental quantification (^{31}P-NMR) expressed as mmol/g, for SWK, HWK, SG, WS_{SE} lignins before and after dansylation and percentage of conversion for aliphatic alcohols, carboxylic acids and phenols.

Chromatographic data elaboration

GPC chromatograms were acquired on acetylated and dansylated specimens as both UV (280 nm) and fluorescence (λ exc: 390 nm, λ em: 550 nm) profiles to gain information about phenols distribution in the examined lignin samples against lignin skeleton response. Fluorescence full spectra of the dansylated lignin samples are reported in Supporting Information. The same approach was used in the elaboration of model compounds chromatograms. A basic assumption was made on the behavior of the molar extinction coefficient and the fluorescence intensity at the variation of the molecular weight: both were considered constant and independent from the species constituting acetylated and dansylated lignin samples. This means that the UV response of lignin and the fluorescence response of dansyl groups linked to aromatic rings was considered not to be affected by changes in the molecular weight and, especially, by the variegated chemical structure offered by lignin.

In this view the four chromatograms obtained after GPC analysis were exploited as follows:

1) The UV profile of a generic acetylated lignin sample was used to determine the representative weight fraction of a specific molecular weight range (ranges b→a taken as follows: >10000 g/mol; 10000-5000 g/mol; 5000-3000 g/mol; 3000-2000 g/mol; 2000-1000 g/mol; 1000-160 g/mol). Accordingly, the total UV absorbance calculated over a specified range $\sum_{n=a}^{b} Abs(n)$ was divided by the total UV absorbance calculated over the whole molecular weight distribution $\sum_{n=160}^{10000} Abs(n)$ according to the equation 1:

$$g\ lignin\ (b \rightarrow a) = \frac{\sum_{n=a}^{b} Abs(n)}{\sum_{n=160}^{10000} Abs(n)} \quad (1)$$

For the model mixtures, the integration was performed on the resolved peaks instead of molecular weight ranges.

2) The fluorescence profile of a generic acetylated lignin sample was used to correct the fluorescence signal of the corresponding dansylated specimen as follows: the acetylated lignin

chromatogram was normalized using the proper weight conversion factor (whose calculation has been described in the previous section) and then subtracted from the fluorescence chromatogram of the analogous dansylated sample to remove the auto-fluorescence contribution of the lignin skeleton. Auto-fluorescence phenomena in lignin are promoted by the occurrence of aromatic structures such as phenylcoumarones and stilbenoids.[20,21] When the auto-fluorescence of non-dansylated lignin samples and model compounds was negligible, this step was skipped.

3) The UV profile of a generic dansylated sample (Supporting Information, in comparison with the corresponding acetylated UV profile) was used to estimate the change in the hydrodynamic volume of the corresponding lignin after dansylation. Since the shift towards higher molecular weights after the insertion of the dansyl group is more marked for low molecular weights species, the integration range 1000-160 g/mol in the acetylated profile was modified into 1250-160 g/mol for the dansylated one to take in account this effect. The other ranges were not modified.

4) The elaboration of the fluorescence profile of a generic dansylated sample provided the representative phenolic molar fraction referred to a specified molecular weight range (ranges b →a are the same listed at point 1). The phenolic molar fractions were obtained according to the following relationship 2:

$$PhOH\ mmol\ (b{\rightarrow}a) = \frac{\sum_{n=a}^{b} Fluo(n)}{\sum_{n=160}^{10000} Fluo(n)}\ X\ PhOH\ mmol\ (tot) \qquad (2)$$

where: $\sum_{n=a}^{b} Fluo(n)$ is the fluorescence intensity over a specified molecular weight range b→ a; $\sum_{n=160}^{10000} Fluo(n)$ is the total fluorescence intensity calculated over the whole molecular weight range; and $PhOH\ mmol(tot)$ is the average content of phenols for a given 1 g sample, as obtained from the ^{31}P-NMR quantification.

The obtained data are meant to be used in the expression 3:

$$PhOH\frac{mmol}{g}(b\rightarrow a) = \frac{mmol\ PhOH\ (b\rightarrow a)}{g\ lignin\ (b\rightarrow a)} \quad (3)$$

a molecular weight range-dependent version of the well-known equation[22] usually exploited for the average assessment of lignin phenolic moieties quantification. Again, for the model mixtures, the integration was performed on the resolved peaks instead of molecular weight ranges.

Application to model compounds

The procedure described so far was applied on simple mixtures obtained from the enzymatic dimerization of two model compounds, vanillyl alcohol and isoeugenol. The aim of this first application was: i) to confirm the reaction selectivity on phenols; ii) to detected the intact dansyl unit on the lignin-like structures; iii) to assess the linear correlation between the phenol content and the dansyl fluorescence. The two mixtures were accordingly analyzed by ^{31}P-NMR and GPC-UV-Fluorescence. Figure 2 displays the ^{31}P-NMR spectra of the mixture obtained after flash-column purification of vanillyl alcohol (**A**) enzymatic coupling. The presence of dimer 5-5' structure (**B**) was confirmed by NMR (proton and carbon spectra are reported in Supporting Information) while the dimer 5-O-4 (**C**) was detected by GPC and ^{31}P-NMR, as well as literature accounts.[23] The phosphorous spectrum highlighted the presence of two peaks related to hydroxyl groups (named Alcohol 1 and Alcohol 2) and three peaks related to phenols. The signals Alcohol 1 and 2 were interpreted as unreacted and dimeric vanillyl alcohol, respectively, whereas the peaks in the range 138-140 ppm were ascribed to 5-5', 5-O-4 dimers (**B** and **C**) and unreacted vanillyl alcohol (monomer **A**, G-OH). The total amount of phenols detected by ^{31}P-NMR analysis was 6.39 mmol/g; the hydroxyls distribution among the species is reported in the table inserted in Figure 2.

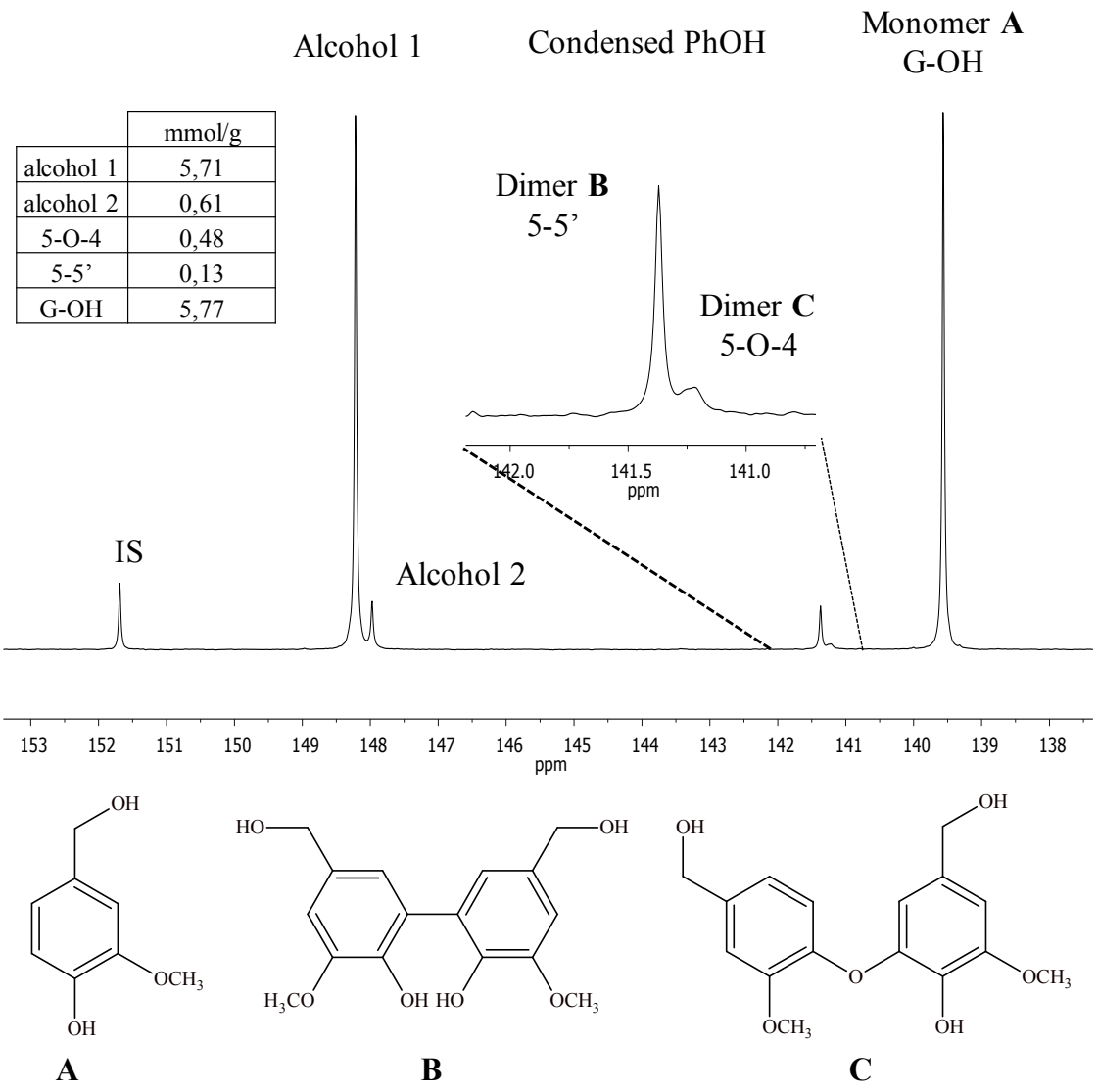


Figure 2. ^{31}P-NMR spectrum and molecular structures of the vanillyl alcohol oxidation products individuated in the Model 1 mixture.

The Model 1 mixture was then dansylated and analyzed by ^{31}P-NMR, which confirmed a 100% dansylation yield of phenols (Supporting Information), and then by GPC. In Figure 3 is reported the GPC-UV profile of the non-dansylated mixture along with the GPC-Fluorescence profile of the mixture after dansylation.

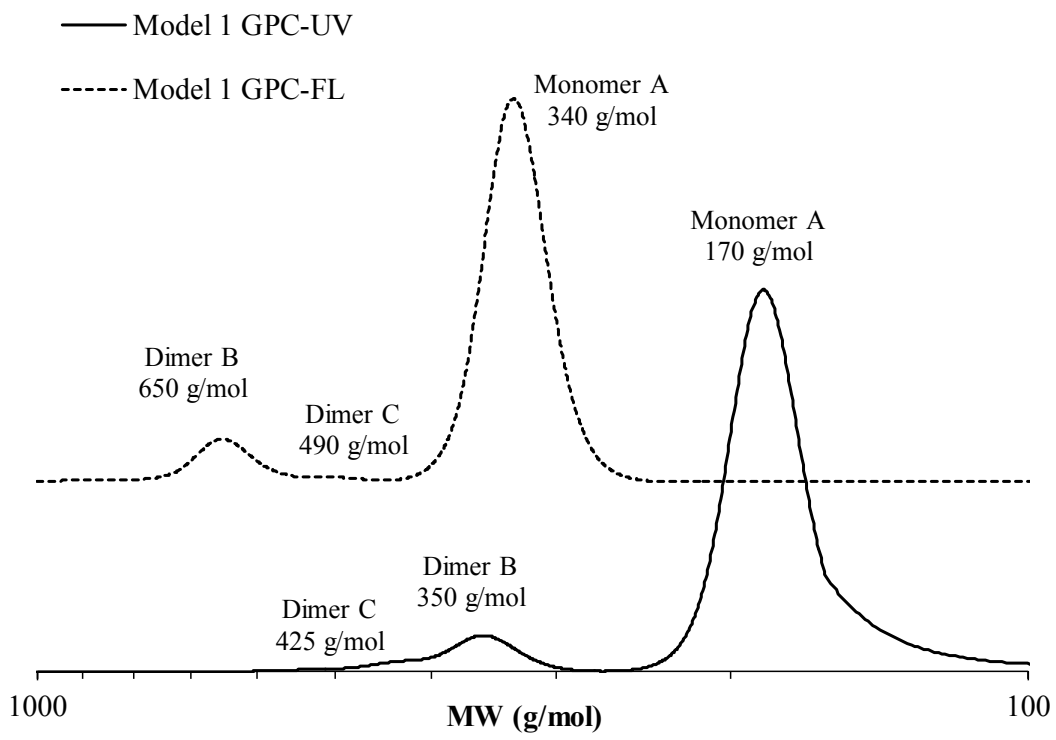


Figure 3. GPC-UV (solid line) and GPC-Fluorescence (dashed line) profiles of the mixture Model 1.

As already found in the ^{31}P-NMR spectrum, the GPC-UV profile (solid line) showed the presence of two dimeric structures (**B** at around 350 g/mol and **C** at around 425 g/mol, visible as a shoulder, assigned to the 5-5' and the 5-O-4 dimer respectively, 306.2 g/mol) generated by the enzymatic dimerization of the monomer (**A**, around 170 g/mol). In the GPC-Fluorescence chromatogram (dashed line) were recognizable: the dansylated vanillyl alcohol (peak **A**, around 340 g/mol), the mono-dansylated 5-O-4 dimer (peak **C,** ca 490 g/mol,) and the di-dansylated 5-5' dimer (peak **B**, ca 650 g/mol). It is worth noticing that the reverse elution order was in line with the stoichiometry of the dansylation reaction, i.e., one dansyl unit for the 5-O-4 dimer (306+233=539 g/mol) *vs* two dansyl units for the 5-5' dimer (306+233+233=772 g/mol).

The results of the elaboration of the chromatographic data, performed as previously described, are reported in Table 2. In the first column, we reported the quantification from ^{31}P-NMR analyses, in the second column the weight fraction from the integration of the GPC-UV chromatogram of un-dansylated mixture, in the third column the PhOH content from the integration of the GPC-FL chromatogram of dansylated material, in the fourth column the PhOH content calculated dividing the PhOH from GPC-FL by the weight fraction and in the last column the theoretical phenol content of each chemical species. Interestingly, through the elaboration of the GPC-Fluorescence profile, the mmol of phenols contained in the different fractions of the mixture were in good agreement with the ^{31}P-NMR ones (compare first and third column, Table 2). After normalization on the weight fraction, the absolute phenol content for each fraction was obtained in sound agreement with the theoretical mmol/g calculated for each structure (compare fourth and fifth column, Table 2).

	PhOH ^{31}P-NMR (mmol/g)	**Weight fraction (w/w)**	**PhOH calc. GPC (mmol)**	**PhOH calculated (mmol/g)**	**PhOH theor. (mmol/g)**
Monomer A	5.78	0.856	5.7	6.7	6.49
Dimer B (5-5')	0.48	0.088	0.5	6.2	6.54
Dimer C (5-O-4)	0.13	0.055	0.1	2.4	3.26

Table 2. GPC-UV and GPC-Fluorescence elaboration output for each fraction of Model 1 mixture: weight fraction (w/w), phenol content (calculated for 1 g of sample) as obtained from GPC-Fluorescence profile elaboration, phenol content in mmol/g and theoretical phenol content (mmol/g). ^{31}P-NMR total phenols content equal to 6.39 mmol/g.

The same procedure was applied to the mixture recovered after flash-column purification of isoeugenol enzymatic coupling. According to GPC and NMR analyses (Supporting

Information), along with unreacted residual monomer (**A**), the main product was the β-5 dimer (Figure 4, dimer **B**).[24] The total amount of phenols detected by ^{31}P-NMR was 4.93 mmol/g; their amount after dansylation was null (Supporting Information).

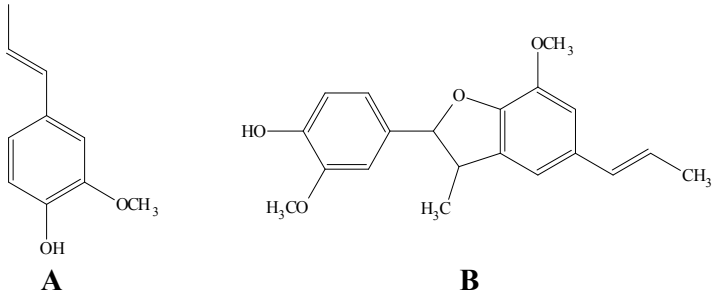


Figure 4. Molecular structure individuated in Model 2.

Figure 5 reports the GPC-UV profile of the non-dansylated mixture along with the GPC-Fluorescence profile after dansylation. In the GPC-UV profile (solid line) was recognizable the peak of the monomer (**A**) at around 200 g/mol, the peak of the β-5 dimer (**B**) at around 400 g/mol and some other unresolved peaks between 600 and 1000 g/mol arising from oligomeric structures (**C**). After dansylation (dashed line), the peaks were shifted to higher molecular weights; their elution order was consistent with the reactivity of uncondensed structures (one dansyl unit per molecule).

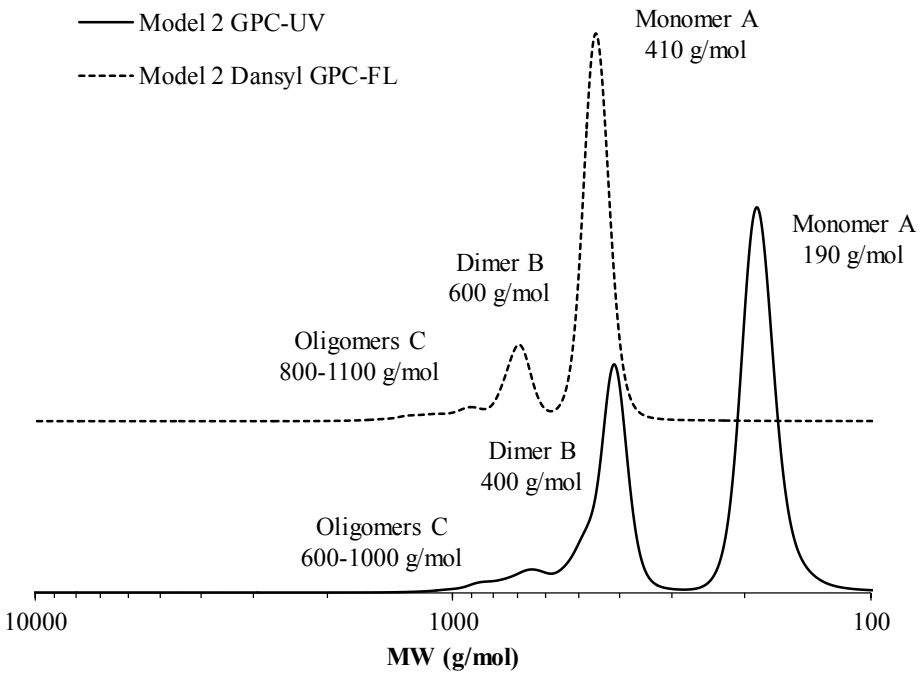


Figure 5. GPC-UV (solid line) and GPC-Fluorescence (dashed line) profiles of Model 2 mixture.

As already occurred for vanillyl alcohol and its dimers, the elaboration of the GPC-UV and GPC-Fluorescence profiles of Model 2 mixture resulted in a phenol quantification associated to monomer, dimers and oligomers that is in good agreement with the calculated theoretical values (Table 3).

	PhOH ^{31}P-NMR (mmol/g)	Weight fraction (w/w)	PhOH calc. GPC (mmol)	PhOH calculated (mmol/g)	PhOH theor. (mmol/g)
Monomer A	Total content: 4.97 (signals overlapped)	0.55	3.4	6.3	6.10
Dimer B (β-5)		0.27	0.9	3.2	3.07
Oligomer C		0.16	0.3	1.9	2.05

Table 3. GPC-UV and GPC-Fluorescence elaboration output for each fraction of Model 2 mixture: weight fraction (w/w), phenol content (calculated for 1 g of sample) as obtained from GPC-Fluorescence profile elaboration, phenol content in mmol/g and theoretical phenol content (mmol/g). ^{31}P-NMR total phenols content equal to 4.97 mmol/g.

In light of the results of these experiments it was possible to confirm that the dansyl unit, even if in presence of intermonomeric lignin-like bonds (such as the 5-5', 5-O-4 and β-5 linkage), is not subjected to quenching phenomena. Moreover, a linear correlation between the dansyl fluorescence and the phenols concentration was found. Both these findings are of importance for the application of the method herein developed.

GPC analyses

GPC profiles of all the examined lignins, under UV-Vis detection (280 nm) after acetylation and under fluorescence detection (λ excitation: 390 nm, λ emission: 550 nm) after dansylation, are reported in Figure 6. In order to confirm the linear correlation between the dansyl fluorescence and the phenols concentration, the integration of GPC-FL profiles of dansylated lignins (in A.U.) and ^{31}P-NMR total phenol content (in mmol/g) for all the samples were compared as reported in Supporting Information, a good linear correlation was found. From a qualitative point of view, the molecular weights distribution of dansylated specimens under fluorescence detection were sharper. In addition, accumulation peaks were diagnostic for co-

elution phenomena of lignin molecules with large phenols content. The molecular weight distributions of both the HWK and WS_{SE} lignin before and after dansylation were similarly scattered along the abscissa while the same chromatograms of the SWK and SG lignin were quite different, characterized by a lower phenols content at high molecular weight. The autofluorescence contribution of the lignin skeleton (profiles not reported) was limited but not negligible for all the samples involved (fluorescence intensity of acetylated *vs* dansylated samples averagely 1:10).

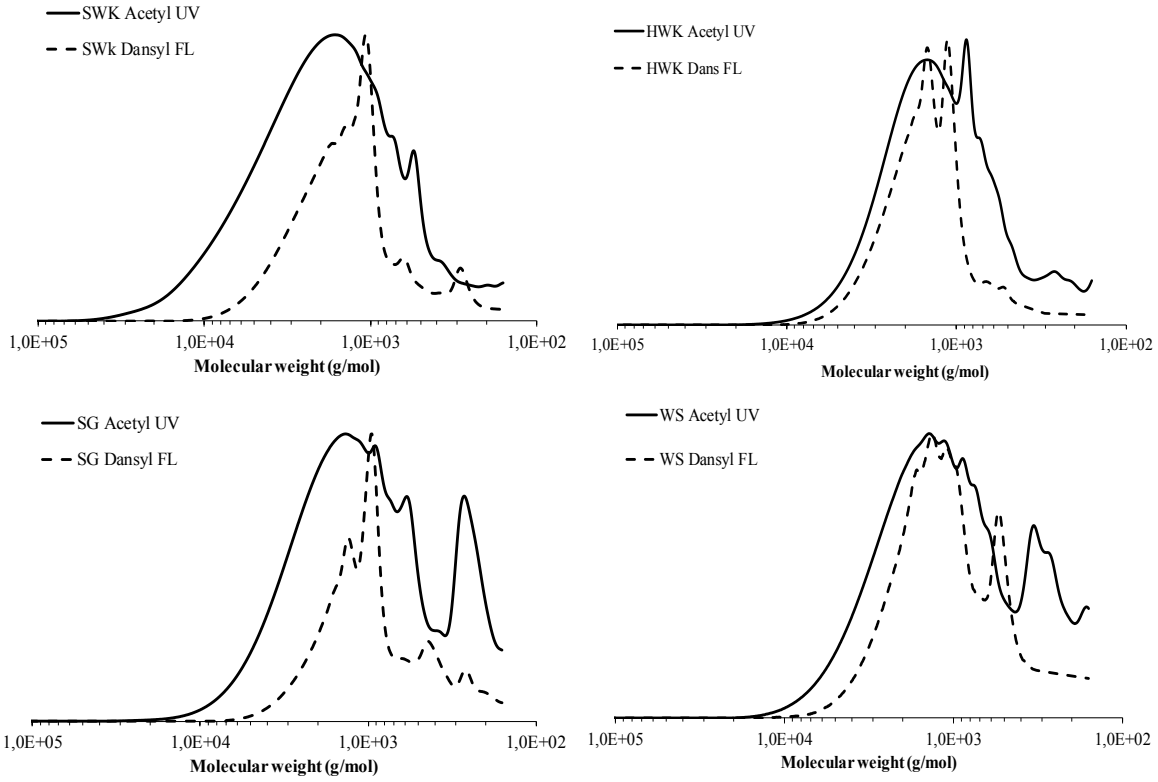


Figure 6. Overlapped GPC profiles of acetylated (280 nm, solid line) and dansylated (exc: 390 nm, em: 550 nm, dashed line) SWK, HWK, SG, and WS_{SE} lignins.

The calculated phenols content for the four technical lignins over different molecular weight ranges is reported in Table 4. These data were extracted from the GPC chromatograms, as

described in the "Chromatographic data elaboration" section. In order to give a complete picture, the average molecular weight indexes and labile -OH groups distribution data obtained by GPC-UV and ^{31}P-NMR analysis are reported.

	SWK	**HWK**	**SG**	**WS_{SE}**
M_n	3030	1720	1730	1700
M_w	7250	2730	3360	3100
PD	2.39	1.59	1.94	1.83
-OH (mmol/g)	2.02	1.12	1.84	2.02
-COOH (mmol/g)	0.52	0.38	1.04	0.68
PhOH (mmol/g)	4.39	2.93	3.71	2.09
mmol/g PhOH per MW range				
< 10000 g/mol	0.1	0.2	0.0	0.2
10000-5000 g/mol	1.2	1.7	0.3	0.6
5000-3000 g/mol	3.0	2.8	1.3	1.2
3000-2000 g/mol	4.6	3.3	2.5	1.9
2000-1000 g/mol	4.3	2.8	3.1	1.9
> 1000 g/mol	7.7	3.0	5.6	2.6

Table 4. GPC outcomes, ^{31}P-NMR characterization and phenol quantification as calculated from the GPC fluorescence profile of SWK, HWK, SG and WS_{SE} technical lignins expressed as mmol/g at the specified molecular weight range (standard error ±0.1 mmol/g).

The same data can be expressed as number of phenols functionality per phenylpropanoidic unit ($PhOH/C_9$, where the molecular weight of the C_9 unit was set to 200 g/mol) to quickly evaluate the amount of phenols contained in each fraction. Moreover, this new set of data can be conveniently visualized in Figure 7.

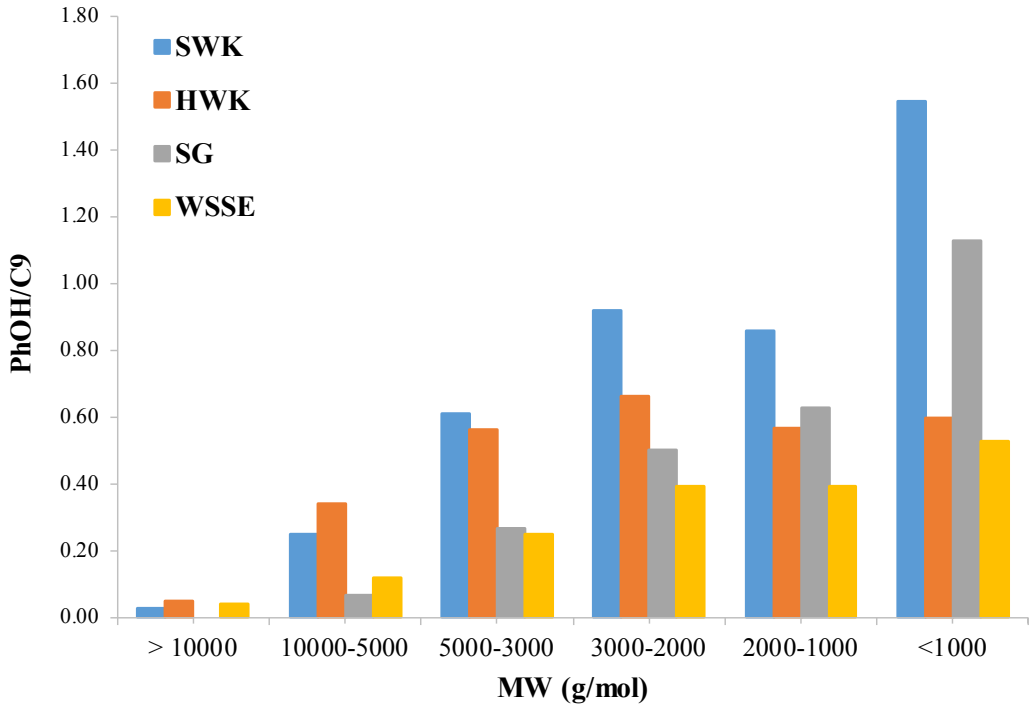


Figure 7. PhOH/C_9 trend for the examined lignin samples SWK, HWK, SG and WS_{SE} over the molecular weight ranges considered.

For the SWK and SG lignins, the phenolic distribution over the examined ranges was quite heterogeneous: at high molecular weights, the number of phenol/C_9 was low, whereas it rapidly increased for low molecular weights.[25] In particular, the behavior of SWK lignin was remarkable in that the phenols content was higher than 1 PhOH/C_9 below 3000 g/mol. It seems like SWK lignin is constituted by different motifs: at higher molecular weight prevails a more native structure, less condensed, whereas at lower molecular weight residual condensed lignin[26] structures not degraded by the process coexist with "new", condensed structures generated *via* radical/ionic coupling of lignin fragments released during the kraft treatment.[4,27,28] In order to explain the unexpected phenolic content below 1000 g/mol, also a partial demethoxylation could be considered. These data for a softwood kraft lignin are in good agreement with the ones reported by Crestini and Lancefield.[4,27] On the contrary, the HWK lignin showed a more

homogenous phenolic distribution, averagely comprised between 0.4-0.6 phenols per C_9 over the whole examined ranges. All the hardwood lignin fractions seemed to be affected by the kraft process at the same extent but the pristine syringyl nature of the macromolecules decreased the formation of new condensed structures during kraft pulping. Lastly, the WS_{SE} lignin displayed a lower phenol content over the whole ranges, and especially at higher molecular weights, that was consistent with a mainly uncondensed structure.[29]

This novel analytical approach offers several valuable insights, for example a tool for researchers to study the lignin chemical modification after extractive processes and to decide the best lignin fraction to select for a particular application. The higher phenolic content at low molecular weights makes the examined softwood kraft lignin a good candidate for an up-grading treatment aimed at the preparation of antioxidant molecules[30], reactive polyfunctional precursors and cross-linkers. On the other hand, the investigated hardwood kraft lignin, characterized by a constant phenolic content over its molecular weight distribution, could be easily used without preliminary fractionation process.

As an application, the phenolic groups distribution was employed in the forecasting of a particular chemo-physical property, the polymer thermal protection, as described by Barana et al.[31] In that paper, the authors found that the ratio between the total phenolic content and the chromatographic peak molecular weight $[PhOH]/M_p$ was linearly correlated to the OIT (Oxygen Induction Time), the protection time measured by DSC (Differential Scanning Calorimetry), on lignin/natural rubber blend. From the data obtained with GPC-FL analyses, for each lignin specimen (SWK, HWK, SG and WS_{SE}) the ratio between the PhOH (b→a) content, expressed as mmol/g, and the average molecular weight of the specific molecular weight range ($\overline{MW}_{b\to a}$=(b+a)/2) was calculated. Then the theoretical OIT was estimated applying the following linear correlation 4:

$$OIT(b\to a) = 11488^{\frac{[PhOH(b\to a)]}{\overline{MW}_{b\to a}}} + 0.2331 \quad (4)$$

and the OIT values obtained were normalized by the weight representativeness of the considered lignin fraction. The sum of these values represented the calculated OITs and they were compared with the experimental ones.[31] The results are reported in Table 5. Calculated and experimental values are in good agreement although a general overestimation is observed for calculated OITs.

		SWK	HWK	SG	WS_{SE}
OIT weighed (min)	> 10000	0.01	0.00	0.00	0.00
	10000-5000	0.24	0.07	0.03	0.04
	5000-3000	1.44	0.78	0.41	0.39
	3000-2000	3.65	2.72	1.76	1.38
	2000-1000	9.54	8.28	7.59	4.84
	<1000	34.54	19.36	43.85	20.48
OIT calculated (min)		**49.42**	**31.20**	**53.64**	**27.13**
OIT experimental (min)		**44.2**	**23.6**	**56.5**	**18.5**

Table 5. Partial, calculated and experimental OITs for SWK, HWK, SG and WS_{SE} lignin.

The data are in line with the results reported in a paper by Sadeghifar et al.[32], where the authors studied the effect of the acetone-based fractionation of SWK on the OIT_{temp} (Oxidation Induction Temperature) of lignin-polyethylene blends. It is worth noticing that the fraction at lower molecular weight (below 2000 g/mol) are responsible, for at least 90% contribution of the whole OIT and this is true for all the lignin examined. Anyway, among the four samples, the most interesting were the SWK and SG lignins that, showing the highest OIT values, provide the best performance allowing for the use of a reduced amount of lignin in the composite. An experimental confirmation of those observation could be found in Barana et

al.[25] where the performance at break of fractionated SWK lignin-natural rubber composites were correlated to their phenol content.

CONCLUSIONS

To conclude, this analytical approach opens several future perspectives. In theory, this approach could be applied to every lignin preparation. Moreover, other characteristic functional groups of lignin, such as alcohols and carboxylic acid, could be investigated to establish a complete correlation between functional groups and molecular weight. The main goal is to build structure-property-application relationships (SPARs) required for any future large-scale application of technical lignins.

SUPPORTING INFORMATION

Model compounds ^{1}H, ^{13}C and ^{31}P-NMR characterization, ^{31}P-NMR spectra of reference (black line) and dansylated (red line) lignins, excitation and emission spectra of dansylated lignins, correlation between dansylated lignin fluorescence and total phenol content, GPC chromatograms of acetylated reference lignins and dansylated lignins (UV and fluorescence profiles) are supplied as Supporting Information.

REFERENCES

(1) Gellerstedt, G., Henriksson, G. Monomers, Polymers and Composites from Renewable Resources. In Monomers, Polymers and Composites from Renewable Resources; Belgacem, M. N., Gandini, A., Eds.; Elsevier: Amsterdam, 2008; pp 201-224, DOI 10.1016/B978-0-08-045316-3.X0001-4.

(2) https://www.prnewswire.com/news-releases/global-lignin-products-market---segmented-by-product-type-source-application-and-geography---trends-and-forecasts-

2015-2020---reportlinker-review-300145371.html.

(3) Del Río, J. C.; Rencoret, J.; Prinsen, P.; Martínez, Á. 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 (23), 5922-5935, DOI 10.1021/jf301002n.

(4) Crestini, C.; Lange, H.; Sette, M.; Argyropoulos, D. S. On the Structure of Softwood Kraft Lignin. Green Chem. 2017, 19 (17), 4104-4121, DOI 10.1039/c7gc01812f.

(5) LUNDQUIST, K.; KIRK, T. K. Fractionation-Purification of an Industrial Kraft Lignin. Tappi 1980, 63 (1), 80-82.

(6) Costa, C. A. E.; Pinto, P. C. R.; Rodrigues, A. E. Lignin Fractionation from E. Globulus Kraft Liquor by Ultrafiltration in a Three Stage Membrane Sequence. Sep. Purif. Technol. 2018, 192 (June 2017), 140-151, DOI 10.1016/j.seppur.2017.09.066.

(7) Zinovyev, G.; Sumerskii, I.; Korntner, P.; Sulaeva, I.; Rosenau, T.; Potthast, A. Molar Mass-Dependent Profiles of Functional Groups and Carbohydrates in Kraft Lignin. J. Wood Chem. Technol. 2017, 37 (3), 171-183, DOI 10.1080/02773813.2016.1253103.

(8) Toledano, A.; Serrano, L.; Garcia, A.; Mondragon, I.; Labidi, J. Comparative Study of Lignin Fractionation by Ultrafiltration and Selective Precipitation. Chem. Eng. J. 2010, 157 (1), 93-99, DOI 10.1016/j.cej.2009.10.056.

(9) Boeriu, C. G.; Fiţigău, F. I.; Gosselink, R. J. A.; Frissen, A. E.; Stoutjesdijk, J.; Peter, F. Fractionation of Five Technical Lignins by Selective Extraction in Green Solvents and Characterisation of Isolated Fractions. Ind. Crops Prod. 2014, 62, 481-490, DOI 10.1016/j.indcrop.2014.09.019.

(10) Wang, K.; Xu, F.; Sun, R. Molecular Characteristics of Kraft-AQ Pulping Lignin Fractionated by Sequential Organic Solvent Extraction. Int. J. Mol. Sci. 2010, 11 (8), 2988-3001, DOI 10.3390/ijms11082988.

(11) Rohde, V.; Böringer, S.; Tübke, B.; Adam, C.; Dahmen, N.; Schmiedl, D. Fractionation of Three Different Lignins by Thermal Separation Techniques-A Comparative Study. GCB Bioenergy 2019, 11 (1), 206-217, DOI 10.1111/gcbb.12546.

(12) Ajao, O.; Jeaidi, J.; Benali, M.; Abdelaziz, O. Y.; Hulteberg, C. P. Green Solvents-Based Fractionation Process for Kraft Lignin with Controlled Dispersity and Molecular Weight. Bioresour. Technol. 2019, 291 (May), 121799, DOI 10.1016/j.biortech.2019.121799.

(13) Cui, C.; Sun, R.; Argyropoulos, D. S. Fractional Precipitation of Softwood Kraft Lignin: Isolation of Narrow Fractions Common to a Variety of Lignins. ACS Sustain. Chem. Eng. 2014, 2 (4), 959-968, DOI 10.1021/sc400545d.

(14) Jääskeläinen, A. S.; Liitiä, T.; Mikkelson, A.; Tamminen, T. Aqueous Organic Solvent Fractionation as Means to Improve Lignin Homogeneity and Purity. Ind. Crops Prod. 2017, 103, 51-58, DOI 10.1016/j.indcrop.2017.03.039.

(15) Jiang, X.; Savithri, D.; Du, X.; Pawar, S.; Jameel, H.; Chang, H. M.; Zhou, X. Fractionation and Characterization of Kraft Lignin by Sequential Precipitation with Various Organic Solvents. ACS Sustain. Chem. Eng. 2017, 5 (1), 835-842, DOI 10.1021/acssuschemeng.6b02174.

(16) Salanti, A.; Zoia, L.; Orlandi, M. Chemical Modifications of Lignin for the Preparation of Macromers Containing Cyclic Carbonates. Green Chem. 2016, 18 (14), 4063-4072, DOI 10.1039/c6gc01028h.

(17) Zoia, L.; Salanti, A.; Tolppa, E. L.; Ballabio, D.; Orlandi, M. Valorization of Side-Streams from a SSF Biorefinery Plant: Wheat Straw Lignin Purification Study. BioResources 2017, 12 (1), 1680-1696, DOI 10.15376/biores.12.1.1680-1696.

(18) Bruschi, M.; Orlandi, M.; Rindone, B.; Rummakko, P.; Zoia, L. Asymmetric Biomimetic Oxidations of Phenols Using Oxazolidines as Chiral Auxiliaries: The

Enantioselective Synthesis of (+)- and (-)-Dehydrodiconiferyl Alcohol. J. Phys. Org. Chem. 2006, 19 (8-9), 592-596, DOI 10.1002/poc.1089.

(19) Cruces-Blanco, C.; Carretero, A. S.; Peinado, S. F.; Gutiérrez, A. F. Spectrofluorimetric Determination of Methyl Paraben in Pharmaceutical Preparations by Means of Its Dansyl Chloride Derivative. Mikrochim. Acta 2000, 134 (1-2), 107-111, DOI 10.1007/s006040070062.

(20) Evdokimov, A. N.; Kurzin, A. V.; Fedorova, O. V.; Lukanin, P. V.; Kazakov, V. G.; Trifonova, A. D. Desulfurization of Kraft Lignin. Wood Sci. Technol. 2018, 52 (4), 1165-1174, DOI 10.1007/s00226-018-1014-1.

(21) Albinsson, B.; Li, S.; Lundquist, K.; Stomberg, R. The Origin of Lignin Fluorescence. J. Mol. Struct. 1999, 508 (1-3), 19-27, DOI 10.1016/S0022-2860(98)00913-2.

(22) Alessandro Granata and Dimitris S. Argyropoulos Dimitris, S. 2-Chloro-4,4,5,5-Tetramethyl-1,3,2-Dioxaphospholaan Reagent for the Accurate Determination of the Uncondensed and Condensed Phenolic Moieties in Lignins. J. Agric. Food Chem 1995, 43, 1538-1544, DOI 10.1021/jf00054a023.

(23) Lahtinen, M.; Heinonen, P.; Oivanen, M.; Karhunen, P.; Kruus, K.; Sipilä, J. On the Factors Affecting Product Distribution in Laccase-Catalyzed Oxidation of a Lignin Model Compound Vanillyl Alcohol: Experimental and Computational Evaluation. Org. Biomol. Chem. 2013, 11 (33), 5454-5464, DOI 10.1039/c3ob40783g.

(24) Salanti, A.; Orlandi, M.; Tolppa, E. L.; Zoia, L. Oxidation of Isoeugenol by Salen Complexes with Bulky Substituents. Int. J. Mol. Sci. 2010, 11 (3), 912-926, DOI 10.3390/ijms11030912.

(25) Barana, D.; Orlandi, M.; Zoia, L.; Castellani, L.; Hanel, T.; Bolck, C.; Gosselink, R. Lignin Based Functional Additives for Natural Rubber. ACS Sustain. Chem. Eng. 2018, 6 (9), 11843-11852, DOI 10.1021/acssuschemeng.8b02145.

(26) Domínguez-Robles, J.; Tamminen, T.; Liitiä, T.; Peresin, M. S.; Rodríguez, A.; Jääskeläinen, A. S. Aqueous Acetone Fractionation of Kraft, Organosolv and Soda Lignins. Int. J. Biol. Macromol. 2018, 106, 979-987, DOI 10.1016/j.ijbiomac.2017.08.102.

(27) Lancefield, C. S.; Wienk, H. J.; Boelens, R.; Weckhuysen, B. M.; Bruijnincx, P. C. A. Identification of a Diagnostic Structural Motif Reveals a New Reaction Intermediate and Condensation Pathway in Kraft Lignin Formation. Chem. Sci. 2018, 9 (30), 6348-6360, DOI 10.1039/c8sc02000k.

(28) Wolter, K. Lignins: Occurrence, Formation, Structure and Reactions; Wiley: Toronto, 1973; Vol. 19, DOI 10.1093/forestscience/19.2.160.

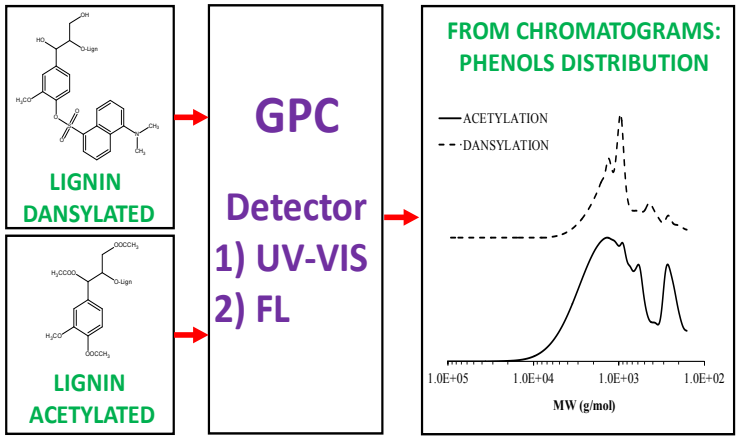
(29) Wang, G.; Chen, H. Fractionation and Characterization of Lignin from Steam-Exploded Corn Stalk by Sequential Dissolution in Ethanol-Water Solvent. Sep. Purif. Technol. 2013, 120, 402-409, DOI 10.1016/j.seppur.2013.10.029.

(30) Arshanitsa, A.; Ponomarenko, J.; Dizhbite, T.; Andersone, A.; Gosselink, R. J. A.; Van Der Putten, J.; Lauberts, M.; Telysheva, G. Fractionation of Technical Lignins as a Tool for Improvement of Their Antioxidant Properties. J. Anal. Appl. Pyrolysis 2013, 103, 78-85, DOI 10.1016/j.jaap.2012.12.023.

(31) Barana, D.; Ali, S. D.; Salanti, A.; Orlandi, M.; Castellani, L.; Hanel, T.; Zoia, L. Influence of Lignin Features on Thermal Stability and Mechanical Properties of Natural Rubber Compounds. ACS Sustain. Chem. Eng. 2016, 4 (10), 5258-5267, DOI 10.1021/acssuschemeng.6b00774.

(32) Sadeghifar, H.; Argyropoulos, D. S. Correlations of the Antioxidant Properties of Softwood Kraft Lignin Fractions with the Thermal Stability of Its Blends with Polyethylene. ACS Sustain. Chem. Eng. 2015, 3 (2), 349-356, DOI 10.1021/sc500756n.

TOC/ABSTRACT GRAPHIC



SYNOPSIS

Information about the phenol distribution as a function of molecular weights in technical lignins is fundamental to understand and completely control a lignin up-grading strategy.