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Structural Characterization of Lignin from Grape Stalks (*Vitis vinifera* L.)

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ABSTRACT: The chemical structure of lignin from grape stalks, an abundant waste of winemaking, has been studied. The dioxane lignin was isolated from extractive- and protein-free grape stalks (*Vitis vinifera* L.) by modified acidolytic procedure and submitted to a structural analysis by wet chemistry (nitrobenzene and permanganate oxidation (PO)) and spectroscopic techniques. The results obtained suggest that grape stalk lignin is an HGS type with molar proportions of *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units of 3:71:26. Structural analysis by ^{1}H and ^{13}C NMR spectroscopy and PO indicates the predominance of *β*-*O*-4′ structures (39% mol) in grape stalk lignin together with moderate amounts of *β*-5′, *β*-*β*, *β*-1′, 5-5′, and 4-*O*-5′ structures. NMR studies also revealed that grape lignin should be structurally associated with tannins. The condensation degree of grape stalks lignin is higher than that of conventional wood lignins and lignins from other agricultural residues.

KEYWORDS: *grape stalks, dioxane lignin, nitrobenzene oxidation, permanganate oxidation, ^{1}H NMR, ^{13}C NMR, HSQC, FTIR, UV−vis*

INTRODUCTION

Winemaking is an important agricultural sector contributing substantially to national economies around the world. Total worldwide wine production is around 262 millions hL, 60% of which is produced by European Union countries.[1] The main wastes generated in the winemaking process (grape pomaces) are skins, pulp, stalks, and seeds, which account for 25−35 kg/hL of produced wine.[2] Grape stalks are one of the major byproducts of the wine industry. On average, 1 hL of wine generates up to 4 kg of grape stalks.[2] The utilization of wine byproducts is gaining increasing attention due to the environmental concerns and potential profitable applications.[3,4] Besides current uses of grape stalks as fertilizers,[5,6] the possibilities of using such materials for the production of biosorbents,[7] dietary fibers,[8] antioxidant supplements,[9] activated carbon,[10] and cellulosic pulp[11] have been demonstrated.

Grape stalks are a lignocellulosic fiber material composed essentially by cellulose, hemicelluloses, lignin, and tannins.[3,4] Similarly to jute, cotton stalks, dhaincha,[12] kenaf (*Hibiscus cannabinus*),[13] and reed (*Arundo donax*),[14] grape stalks may be considered an alternative to wood sources of fibers for papermaking and biocomposites.[4,15] Carbohydrates contribute to >50% of grape stalks,[4,16] the most abundant being cellulose (ca. 30−35%) and *O*-acetyl-4-*O*-methylglucurono-*β*-D-xylan (ca. 10−15%). One specific feature of grape stalks is the high content of tannins, their amount being comparable to the lignin content.[4] Grape stalks contain several classes of polyphenolic compounds, such as phenolic acids, flavonols, and flavanonols. The major part of tannins consist of polymeric proanthocyanidins composed of (−)-epicatechin units together with smaller amounts of (+)-catechin, (−)-epicatechin gallate, and (−)-epigallocatechin.[17] Among other polyphenolic compounds, quercetin 3-glucuronide, catechin, caffeoyltartaric acid, and dihydroquercetin 3-rhamnoside (astilbin) have been detected.[17]

Often, condensed tannins are determined together with lignin in the acid-insoluble residue called Klason lignin, thus overestimating its content by almost twice.[4] The accurate determination of Klason lignin is possible only after the preliminary removal of extractives, proteins, and condensed tannins from grape stalks. The Klason lignin content in grape stalks corrected for extractives, proteins, and tannins is around 17%.[4] Despite increased research activity on the chemical processing of grape stalks, practically no reliable information on the chemical structure of lignin is available. However, the undefined structure of lignin and/or its possible association with tannins was suggested to be a reason for the extremely difficult delignification of grape stalks by oxygen-alkali and organosolv,[11] soda,[18] and kraft[4] pulping.

The main goal of this work was the structural evaluation of lignin isolated from grape stalks by a soft acidolytic technique (dioxane lignin). Lignin was characterized by wet chemistry (nitrobenzene and permanganate oxidation) and spectroscopic techniques (1D and 2D NMR, FTIR, and UV spectroscopy). The molecular weight was assessed by size exclusion chromatography (SEC).

MATERIALS AND METHODS

Preparation of Plant Material. Grape stalks from red grape pomace (*Vitis vinifera*) were collected after destemming at the Quinta do Serrado in Penalva do Castelo, Region of Dão (Portugal). The material was dried at room temperature, milled on a cross-beater mill SKI (Retsch, Haan, Germany), and sieved to 0.5−1.0 mm sawdust. The grape stalks were submitted to Soxhlet extraction with acetone (6 h) to remove extractives, according to Tappi standard T 204 om-88. Proteins were removed by the treatment of the extractives-free grape

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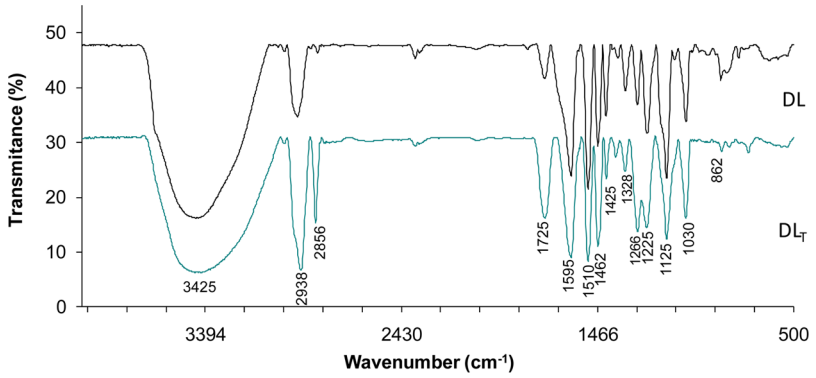
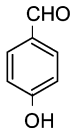
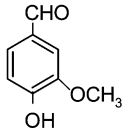
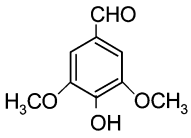


Figure 1. FTIR spectra of dioxane lignins isolated from grape stalks before (DL_T) and after (DL) removal of tannins.

Table 1. Results on Nitrobenzene Oxidation of Dioxane Lignins before (DL_T) and after Tannin Removal (DL) and Lignin of Grape Stalks

characteristic product	structural unit	% molar		
		DL	DL_T	grape stalks
 *p*-hydroxybenzaldehyde	**H**	1	2	3
 Vanillin	**G**	69	73	71
 Syringaldehyde	**S**	30	25	26

stalks, with a 1% pepsin solution in 0.1 M HCl at 40 °C overnight, followed by hot water washing until neutral reaction of filtrate.[19] The tannins content was assessed in extractives- and proteins-free grape stalks by reflux with 0.3% NaOH solution (liquid-to-solid ratio of 100:1) under nitrogen atmosphere for 1 h. The lignin content was determined by the Klason method according to Tappi standard T 222 om-88, using grape stalks free of extractives, proteins, and tannins. The Klason lignin content in the grape stalks was 17.4%.

Isolation of Lignin. The lignin was isolated by acidolytic procedure from grape stalks free of extractives and proteins (DL_T) and from grape stalks free of extractives, proteins, and tannins (DL), according to the previously described method.[20] Typically, 10 g of dry extractives- and proteins-free grape stalks sawdust (0.5−1.0 mm) was submitted to three sequential extractions (each 60 min) with 500 mL of a dioxane/water solution (9:1, v/v) containing 0.1 M HCl, under a nitrogen atmosphere. The resulting extracts were further concentrated separately to a volume of ca. 150 mL. Finally, lignin was precipitated in distilled cold water (1.5 L), centrifuged, and washed with distilled cold water until filtrates were of neutral pH and vacuum-dried at room temperature (ca. 20 °C). Alternatively, another sample of dioxane lignin (DL) was isolated from extractives- and proteins-free grape stalks after additional treatment with 0.3% NaOH solution under nitrogen atmosphere, aiming to eliminate the concomitant condensed tannins.[4] The yields of DL_T and DL were about 70−75% of total lignin content in grape stalks (Klason lignin = 17.4%).

Chemical Analyses. The lignin in grape stalks sawdust and isolated dioxane lignins were characterized by oxidation with nitrobenzene under alkaline conditions, according to the previously described procedure.[21] The oxidation products were dissolved in 200 μL of pyridine and analyzed by GC-MS on a Trace Gas Chromatograph 2000 equipped with a Thermo Scientific DSQII mass spectrometer (Thermo Fisher Scientific, Austin, TX, USA). The conditions of GC analysis were as follows: column capillary, 30 m × 0.32 mm i.d. 0.25 μm, DB-1 (Agilent J&W, Santa Clara, CA, USA); initial temperature of the column, 100 °C; gradient of temperature, 4 °C/min; final temperature of the column, 270 °C; temperature of the injector, 240 °C; and temperature of the detector, 250 °C.

The permanganate oxidation of isolated lignin with tannins (DL_T) was performed as described previously.[22] The ethylated oxidation products were identified by GC-MS using the equipment as indicated above. The chromatographic conditions were as follows: initial temperature of the column, 180 °C; gradient of temperature, 4 °C/min; final temperature of the column, 290 °C; injector temperature, 250 °C.

Methoxy groups in lignins were analyzed according to the Zeisel−Vieböck−Schwappach method.[23]

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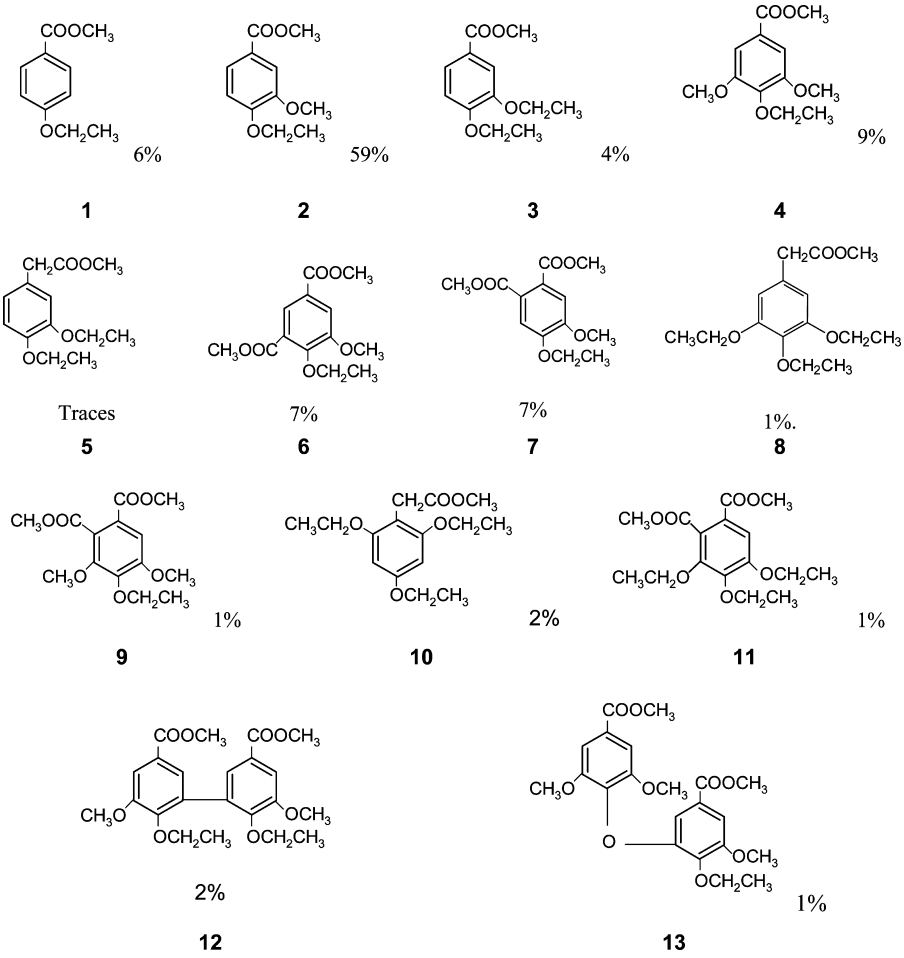

Figure 2. Frequency of occurrence (% mol) of carboxylic acid methyl esters obtained from oxidation of dioxane lignin (DL_T) with potassium permanganate.

Analysis by UV and FTIR Spectroscopy. Ultraviolet (UV) spectra of dioxane-lignin were recorded in 2-methoxyethanol on a JASCO V-560 UV−vis spectrophotometer (Jasco International, Tokyo, Japan), using 1 cm thick quartz cells. The FTIR spectrum of the dioxane-lignins was obtained on a Mattson 7000 spectrometer (Mattson Instruments, Madison, WI, USA), with a resolution of 4.0 cm^{-1} and 132 scans recorded in the medium-infrared area, which extends from 4000 to 400 cm^{-1}.

NMR Analyses. All NMR spectra were registered on an AVANCE 300 spectrometer (Bruker, Wissembourg, France). The ^1H NMR spectra of acetylated dioxane lignins dissolved in chloroform-d_1 (concentration ca. 3%), placed in a 5 mm diameter tube, have been carried out by operating at 300.1 MHz (298 K). The acquisition parameters were as follows: 12.2 μs pulse width (90°), 3 s relaxation delay, and 300 scans.

The quantitative ^{13}C NMR spectrum of dioxane lignin (DL) was recorded by operating at 75.47 MHz (318 K) with TMS as internal reference. DL was dissolved in DMSO-d_6 (ca. 25% concentration) and placed into 10 mm diameter tubes. The acquisition parameters were as follows: 4.1 ms pulse width (90° pulse angle), 12 s relaxation delay, 16K data points, and 20000 scans. The same lignin sample in DMSO-d_6 was placed into a 5 mm diameter tube, and 2D NMR spectra were recorded. The phase sensitive ^1H-detected heteronuclear single-quantum coherence (HSQC) spectrum was acquired over an F1 spectral weight of 12000 Hz and an F2 width of 2000 Hz with a 2048 × 1024 matrix and 128 transients per increment. The delay between scans was 2 s and the delay for polarization transfer was optimized for $^1J_{C-H}$ = 148 Hz. The heteronuclear multiple-bond correlation (HMBC) spectrum was recorded using a coupling evolution time of 110 ms ($^3J_{C-H}$ = 4.5 Hz).

Analysis by Size Exclusion Chromatography (SEC). Two dioxane lignins were analyzed by SEC using a PL-GPC 110 apparatus (Polymer Laboratories, Shropshire, UK), equipped with a precolumn, 10 μm, Plgel, and two 300 mm × 7.5 mm columns, 10 μm, Plgel MIXED D (Polymer Laboratories) and a refraction index detector. Columns and the injection system were maintained at a temperature of 70 °C. The eluent flow (0.1 M LiCl in DMF) was 0.9 mL/min. The lignin solutions (ca. 1%) were prepared just before the analysis by dissolution in eluent solution (0.1 M LiCl in DMF). The column calibration was carried out using lignin model compounds (monomers, dimers, and tetramers) and selected lignin samples (Mp = 950−3200 Da) previously characterized by ESI-MS.[24]

RESULTS AND DISCUSSION

Molecular Weight and Purity of Isolated of Dioxane Lignins. The molecular weights (M_w) of dioxane lignins free of extractives and proteins (DL_T) and free of extractives, proteins, and tannins (DL) were very close to each other (ca. 2600 Da). As could be expected, the removal of condensed tannins from grape stalks before the isolation of dioxane lignin led to much less contaminated lignin sample (DL). This fact was confirmed by FTIR spectroscopy and by analysis of methoxyl group content. Thus, the FTIR spectrum of DL_T showed typical strong signals for tannins and tannic acids at 2920/2860 cm^{-1} (sym/antisym C−H stretching in CH_2), 1718 cm^{-1} (C═O stretching in ketones), and 1610−1600 cm^{-1} (bending vibrations of aromatic ring),[25,26] which were drastically diminished in the spectrum of DL (Figure 1). The methoxyl

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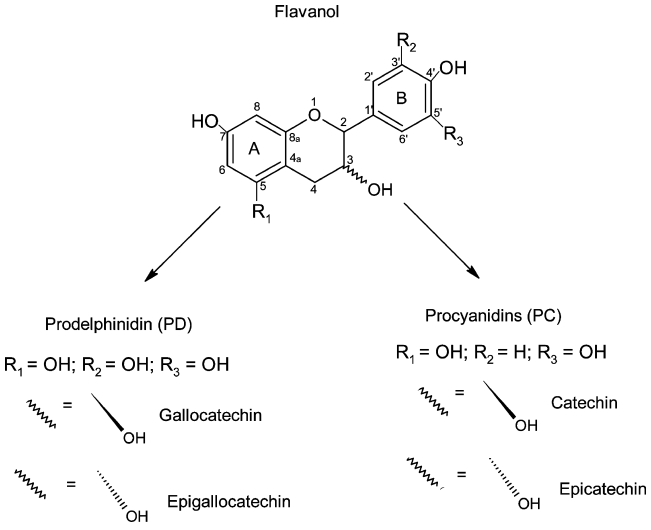

Figure 3. Monomeric units of representative flavanols constituting condensed tannins.

group content was 15.6% for DL_T and 17.3% for DL, which is consistent with the conclusion on the stronger contamination of the former with tannins. Because DL is purer than DL_T, the former is preferable for the qualitative and quantitative characterization by spectroscopic techniques. At the same time, the fact of preliminary alkaline extraction of grape stalks to remove tannins may cause the removal of the alkali-soluble lignin fraction. Hence, DL_T may provide some additional structural information when analyzed by degradation techniques.

FTIR and UV Spectroscopy. DL had FTIR spectrum patterns (Figure 1) typical for lignins constituted by guaiacyl (G) and syringyl (S) units.[27] Thus, besides characteristic aromatic ring bands at 1595, 1510, and 1425 cm^{-1} (aromatic skeletal vibrations in G and S rings), the bands at 1328 and 1266 cm^{-1} were detected and assigned to syringyl and guaiacyl rings breathing with $Ar-OCH_3$ stretching, respectively.[28] The shoulder at ca. 1636 cm^{-1} in the FTIR spectrum indicated the presence of lignin structures with C=C bonds in the propane chain conjugated to an aromatic ring.[29]

The UV spectrum of DL revealed the bands typically observed for dioxane lignins constituted by *p*-hydroxyphenyl (H), syringyl (S) and guaiacyl (G) units (HGS lignins) from kenaf[13] and banana plant.[30] The extinction coefficients (ε) at 212, 240, 284, and 320 nm were 54.8, 19.1, 9,8 and 5.7 L/g/cm, respectively. Attention is drawn to the bathochromic shifted band at 284 nm of relatively low intensity (ε = 9.7 L/g/cm) corresponding to the $\pi \rightarrow \pi^*$ electron transition in the aromatic ring, which usually appears in lignins at ca. 280 nm. Similar features were previously observed in dioxane lignin of kenaf bark contaminated by condensed tannins.[13] The relatively strong absorption at 320 nm could be assigned to the $n \rightarrow \pi^*$ transition in C=O groups conjugated to aromatic moieties and to $\pi \rightarrow \pi^*$ electronic transition in the aromatic ring conjugated with C=C moieties.[13,29]

Nitrobenzene Oxidation. Analysis of nitrobenzene oxidation (NO) products from DL, DL_T, and grape stalks was carried out to assess the molar ratio of structural units in lignin. It was evident that the grape stalk lignin is of HGS type with a relatively high proportion of G units and a small amount of H units (Table 1). H:G:S ratios were slightly different in terms of H unit abundance when NO was carried out on DL/DL_T or directly using grape stalk material (Table 1). This is especially notable for DL, which revealed a low content of H units. This can be tentatively explained by substantial alkaline hydrolysis of *p*-coumaric acid type structures during removal of condensed tannins prior to isolation of DL. The H:G:S ratio obtained by nitrobenzene oxidation of grape stalks was 3:71:26 and may be considered as the most credible because it was assessed on in situ lignin without modification during the isolation procedure.

By comparisin of the H:G:S ratios found in DL and DL_T, it may be proposed that during alkali pre-extraction of grape stalks, before the isolation of dioxane lignin, a small fraction of lignin rich in H and G structural units was removed from the starting material.

Permanganate Oxidation. The information about different "condensed" and "noncondensed" structures was assessed by analysis of products arising from permanganate oxidation (PO) of dioxane lignin isolated from extractives- and proteins-free grape stalks without preliminary alkaline extraction to remove condensed tannins (DL_T sample). DL_T was ethylated prior to oxidation, which allowed protection of the phenolic units against aromatic ring degradation and, at the same time, to distinguish PO products from lignin and structurally associated tannins, thus elucidating their eventual origin.

Detected and identified PO products and their frequencies of occurrence are summarized in Figure 2. Nearly 95% of PO products were identified, most of them belonging to lignin. Thus, products **1**, **2**, and **4** were assigned to noncondensed lignin structures derived from the *p*-hydroxyphenyl (**1**), guaiacyl (**2**), and syringyl (**4**) structural units, respectively. The isohemipinic acid methyl ester (**6**) originated from degraded phenylcoumaran (β-5′) structures, and the products **7** and **9** can be assigned to guaiacyl and syringyl units, respectively, linked by β-6′ and γ-*O*-α bonds (phenylisochroman type structures) or to some other type of still unknown condensed structures. The oxidation products **12** and **13** are derived from biphenyl-type structures (5-5′) and diaryl ether type (4-*O*-5′) structures, respectively. Attention is drawn to the high content of condensed guaiacyl units (products **6** and **7**), which are much more abundant than in lignins of such agricultural stuffs as kenaf,[13] reed, and [14] banana plant[30] or in hardwoods.[20,31] When the H:G:S ratio in DL_T (2:73:25) and the fact that only phenolic units are accessible for the PO analysis are taken into account, the relatively low abundance of syringyc acid methyl ester (**4**) among PO products (Figure 2) indicates that the lignin molecules constituted by syringic units should be much less ramified that those constituted of guaiacyl units. The ratio of uncondensed structural units (products **1**, **2**, and **4**) and the condensed units (products **6**, **7**, **9**, **12**, and **13**) was 64:21 (Figure 2), indicating a relatively higher degree of lignin condensation in grape stalks.

Rather abundant PO products **3**, **5**, **8**, and **10** belong to condensed tannins that are present in grape stalks. The same products were found previously in PO analysis of condensed tannins extracted from pine bark.[32] Thus, products **3** and **5** are derived from the B-ring of procyanidins and proanthocyanidins (Figure 3), which are the main types of condensed tannins in grape stalks.[4,11,17] The PO product **8** is derived from the B-ring of prodelphinidins and indicates the presence of this type of flavonoid in grape stalks. Accordingly, product **10** (Figure 2) is derived from the A-ring of the corresponding flavonoid units

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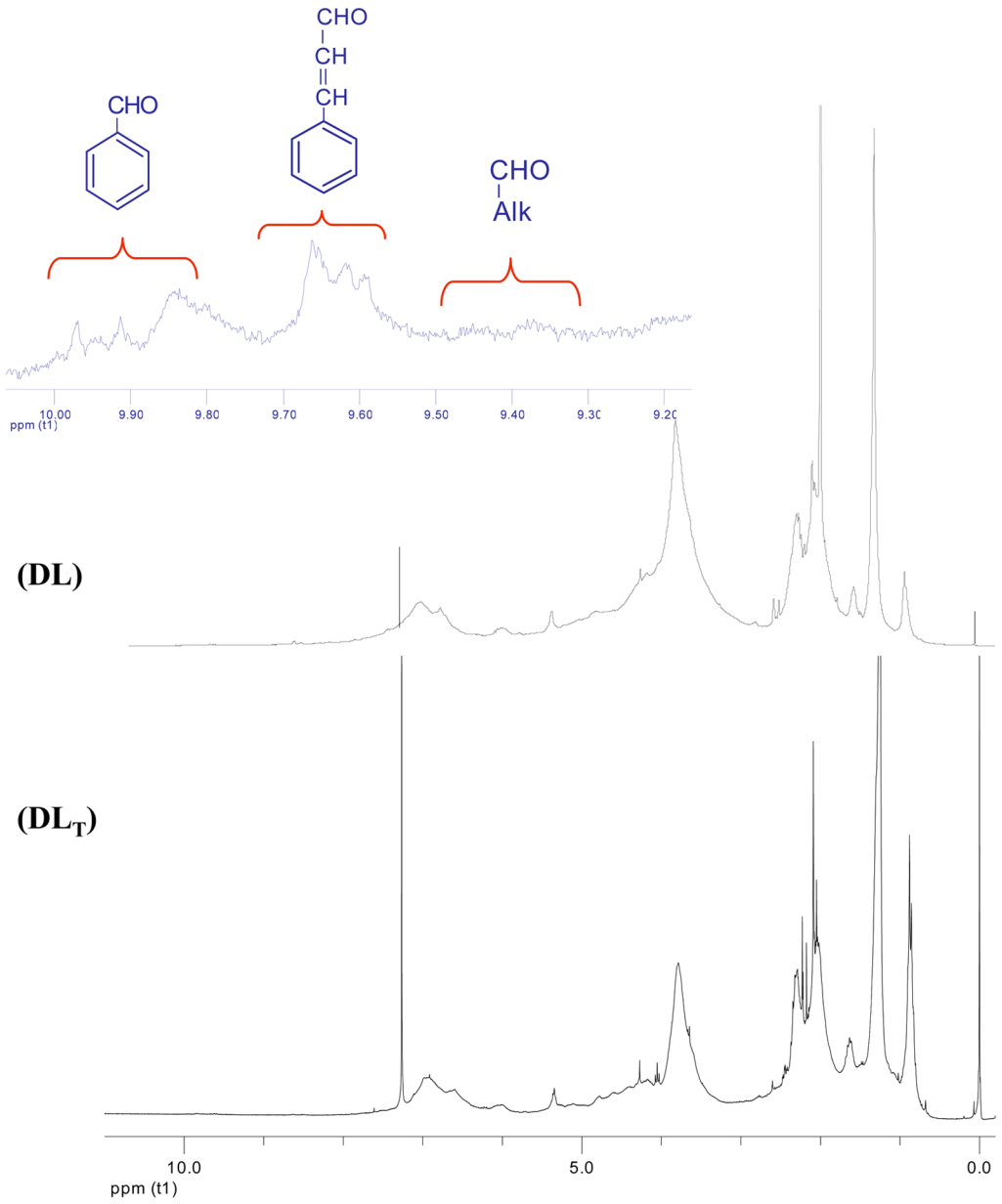

Figure 4. ^{1}H NMR spectrum (DMSO-d_6) of dioxane lignins (DL and DL$_T$) from grape stalks. Expanded range from 9 to 10 ppm shows resonances of formyl protons.

Table 2. Estimation of Frequency of Functional Groups and Major Interunit Linkages in Grape Stalk Dioxane Lignins DL$_T$ and DL As Determined by ^{1}H NMR Spectroscopy

	abundance/100 C_9	
structural element	DL$_T$	DL
OCH_3 (3.5−4.0 ppm)	123	129
aliphatic OH (CO−C$\underline{H}_3$ at 1.8−2.1 ppm)	80	85
phenolic OH (CO−C$\underline{H}_3$ at 2.1−2.5 ppm)	58	47
H_α in β-*O*-4 structures (5.8−6.3 ppm)	34	38
aromatc H (6.3−6.7 ppm; 6.7−7.3 ppm; 7.3−7.9 ppm)	205	212
CHO in benzaldehyde-type structures (9.8−10.0 ppm)	2	1
CHO in cinnamaldehyde-type structures (9.5−9.7 ppm)	2	2
CHO nonconjugated (9.3−9.5 ppm)	trace	trace

(Figure 3). Compound **11** was assigned to the PO product derived from ellagic acid originating from ellagitannins.

^{1}H NMR Spectroscopy. The amounts of hydroxyl and aldehyde groups and some interunit linkages were estimated from the ^{1}H NMR spectrum of acetylated DL. These data were compared with those obtained for DL$_T$ (Figure 4). The quantitation was carried out per phenyl propane unit (PPU) according to the previously defined methodology using the

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Table 3. Quantitative Estimation of Different Structural Elements in DL from Grape Stalks by ^{13}C NMR Spectroscopy (Figure 5)

structure element	calculation[a]	abundance/ C_6
β-O-4′	$I61.0{-}59.0 \times 6/(I_{156.0-102.0} - I_{139.5-138.5} - I_{125.7-124.0} - I_{128.0-127.4})$	0.39
β-5′	$I_{53.3-52.5} \times 6/(I_{156.0-102.0} - I_{139.5-138.5} - I_{125.7-124.0} - I_{128.0-127.4})$	0.03
β-β′	$I_{54.0-53.3} \times 6/(I_{156.0-102.0} - I_{139.5-138.5} - I_{125.7-124.0} - I_{128.0-127.4})$	0.06
β-1′ + *β-5′*	$I_{64.0-62.0} \times 6/(I_{156.0-102.0} - I_{139.5-138.5} - I_{125.7-124.0} - I_{128.0-127.4})$	006
Ar-O	$I_{156.0-140.0} \times 6/(I_{156.0-102.0} - I_{139.5-138.5} - I_{125.7-124.0} - I_{128.0-127.4})$	2.20
Ar-C	$I_{140.0-126.0} \times 6/(I_{156.0-102.0} - I_{139.5-138.5} - I_{125.7-124.0} - I_{128.0-127.4})$	1.50
Ar-H	$I_{124.0-102.0} \times 6/(I_{156.0-102.0} - I_{139.5-138.5} - I_{125.7-124.0} - I_{128.0-127.4})$	2.30
OCH_3	$I_{57.5-54.4} \times 6/(I_{156.0-102.0} - I_{139.5-138.5} - I_{125.7-124.0} - I_{128.0-127.4})$	1.27
S/G	$S/G = (I_{109.0-102.0} \times 3)/(I_{124.0-109.0} \times 2)$	0.56
H units	$I_{163.0-160.0} \times 6/(I_{156.0-102.0} - I_{139.5-138.5} - I_{125.7-124.0} - I_{128.0-127.4})$	0.02
S:G:H		34:64:2

[a] $(I_{139.5-138.5} + I_{125.7-124.0} + I_{128.0-127.4})$ − integral intensities from solvent contaminants.

resonance of methoxy groups at 3.6−4.0 ppm as an internal standard and integrating the proton intensities in the corresponding functionalities.[20] The average number of methoxy groups per PPU was calculated on the basis of the H:G:S ratio determined by NO in grape stalks and dioxane lignins (Table 1).

As was already highlighted by FTIR spectroscopy (Figure 1), the elimination of tannins from grape stalks by alkaline extraction before the lignin isolation by acidolytic technique allows much more pure lignin sample. This proposition was additionally confirmed by ^1H NMR showing the decrease of resonances at 0.7−1.8 ppm assigned to aliphatic moieties in extractives and the decrease of phenolic groups (OH_{ph}) in DL after tannin removal by alkaline extraction before the lignin isolation (Table 2). However, the amount of phenolic groups (OH_{ph}) in DL was still high (47/100 PPU) when compared to the amount of OH_{ph} usually reported in dioxane lignins (20−30/100 PPU).[20,31,33] This fact indicates that tannins still occur in this sample, which correlates with conclusions drawn by analysis of the UV−vis spectrum of DL suggesting the contamination of isolated lignin with condensed tannins.

The relatively low amount of *β-O-4′* structures detected in DL and DL_T (0.34−0.38/PPU), based on the H_α resonance in the corresponding structures at 5.8−6.3 ppm, may be explained by the significantly higher proportion of condensed structures (Table 3). The last proposition correlates with discussed data on PO analysis (Figure 2) and the relatively low content of aromatic protons per PPU detected in proton spectra of dioxane lignins (Table 3).

The amount of benzaldehyde- and cinnamaldehyde-type structures, calculated on the basis of the integral of formyl proton signals in the corresponding structures (Figure 4), was rather insignificant and similar in both studied lignins. Some lower amount of benzaldehyde type structures in DL than in DL_T may be explained by their partial removal during the alkaline extraction of grape stalks before lignin isolation.

^{13}C NMR Spectroscopy. The quantification of the main structures in grape stalk lignin was carried out by quantitative ^{13}C NMR spectroscopy of DL (Figure 5). The resonance assignments were done on the basis of the $^1H-^{13}C$ correlation

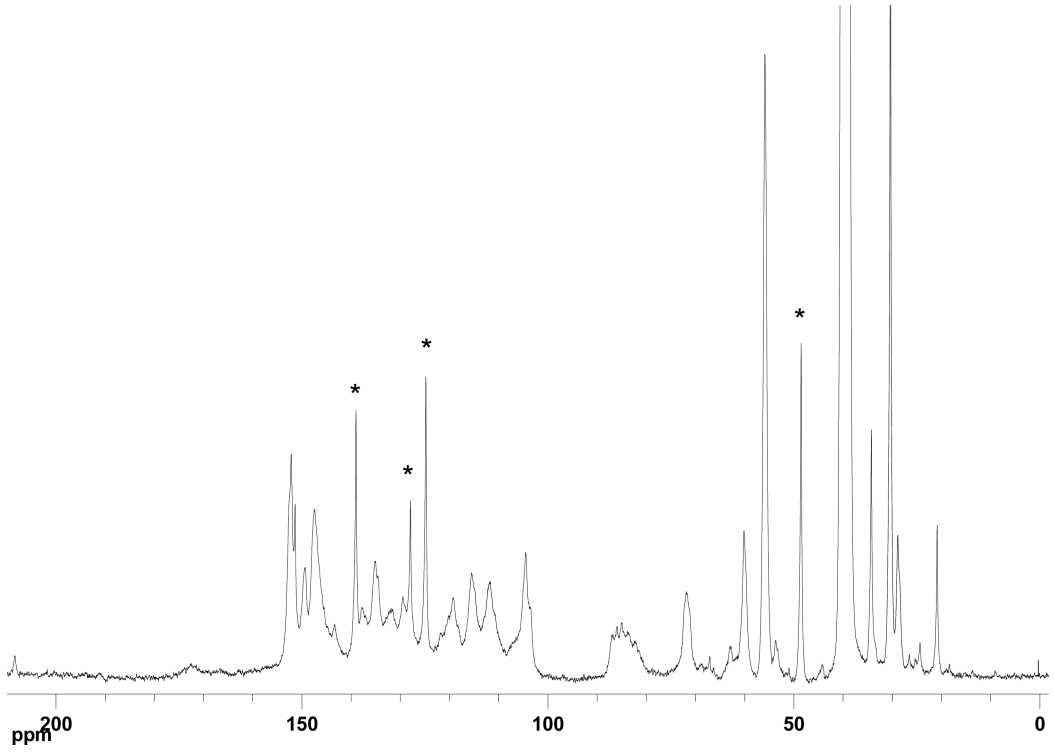

Figure 5. Quantitative ^{13}C NMR spectrum of dioxane lignin (DL) from grape stalks (DMSO-d_6). Solvent contaminants are marked by asterisks.

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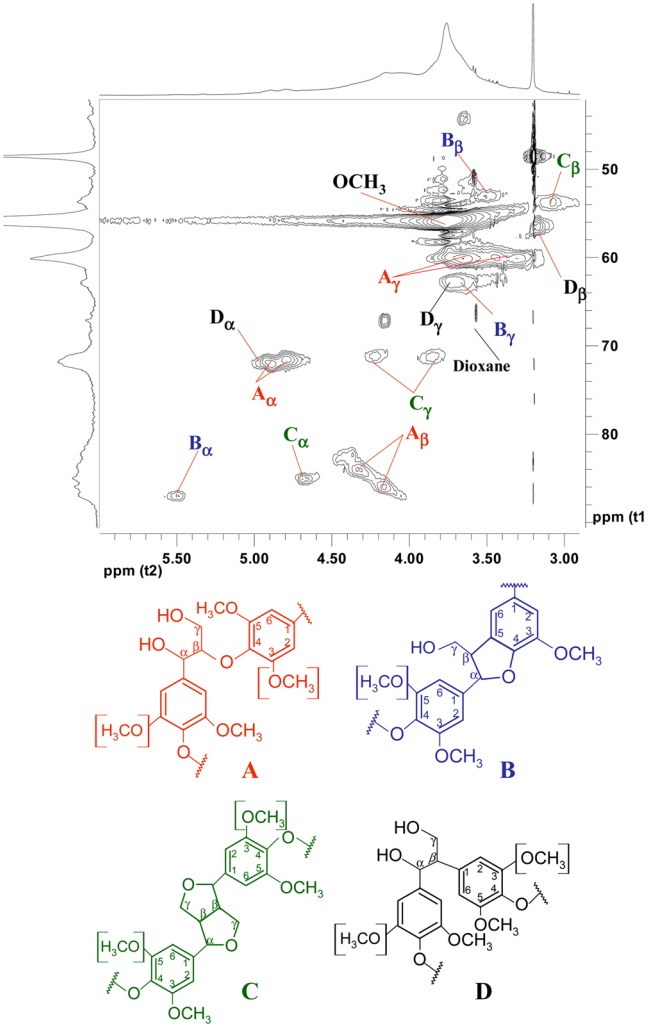

Figure 6. Oxygenated aliphatic region of HSCQ spectrum of DL from grape stalks. Major identified lignin structures, for which assignments are depicted in the spectrum, are presented below.

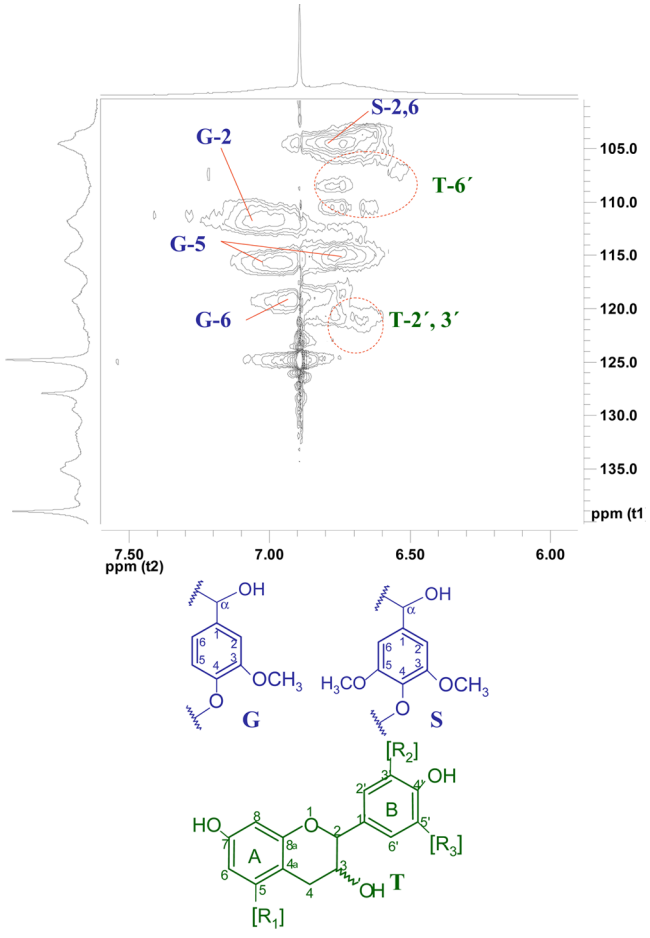

Figure 7. Aromatic region of HSCQ spectrum of DL from grape stalks. Identified guaiacyl (G), syringyl (S), and tannin (T) structures are depicted below.

(HSQC) spectra (Figures 6 and 7) using the well-known databases on lignin[34−36] and lignin model compounds.[37,38]

The primary analysis of the quantitative ^{13}C NMR spectrum of DL revealed unusual resonances for lignins in the range of 20−35 ppm assigned to aliphatic carbons (CH_3, CH_2, and CH) in extractives that were not eliminated during exhaustive extraction of grape stalks with acetone. The presence of condensed tannins was suspected on the basis of the unusually strong resonances for lignin at ca. 146 ppm (Figure 5) assigned to aromatic carbons in free phenolic moieties of proanthocyanidins.[39−41] In addition, the HSQC spectrum of DL (Figure 7) revealed $^{1}H-^{13}C$ correlation cross-peaks typical for the B-ring of procyanidins.[39−41] The cross-peaks typical for the A-ring of procyanidins were of a very low intensity, probably due to their lability under alkaline conditions[25,32,42] and the formation of a series of new structures difficult to assign. Due to the alkaline extraction of grape stalks at 100 °C before the isolation of DL, at least part of the procyanidins associated with DL might be in the form of catechinic acid arising as a result of intramolecular rearrangement reactions under alkaline conditions.[25,42] The notable resonance at ca. 208 ppm, assigned to ketone moieties in catechinic acid,[42] supports this proposition.

The fact of structural association of lignin with condensed tannins in grape stalk lignin supports earlier proposed chemical bonding between them.[4,11] The incorporation of tannins in a lignin network has been proposed in wood[43,44] and recently suggested in coconut coir[45] and in wheat straw[46] lignins. Hence, the formation of a lignin−tannin network could be realized also in the case of grape stalks. Besides this "natural" association, the new bonds could be formed between lignin and tannins during alkali pre-extraction of grape stalks and during acidolytic isolation of DL as depicted in Figure 8. The condensation of lignin with flavanols may explain the unusually high amounts of condensed structures detected in lignin by PO analysis (Figure 2). The cross peak at 44.3/3.62 ppm in the HSQC spectrum of DL (Figure 6) is very close to the corresponding carbon/proton chemical shifts reported for the C-2/H-2 condensed structures resulting from the reaction of procyanidins with phloroglucinol.[25] Unfortunately, the reliable identification of these lignin−tannin condensed structures using HMBC spectra was impossible due to their relatively low abundance in grape stalk lignin.

The quantification of major lignin structure has been done per aromatic ring (C_6) according to a previously described methodology.[20,30] The basic calculations involved are presented in Table 3, where the integral intensities of solvent contaminants were subtracted from intensities of carbons in the aromatic region at 102−156 ppm. Thus, the abundance of lignin structures linked by *β-O-4′* bonds was calculated on the

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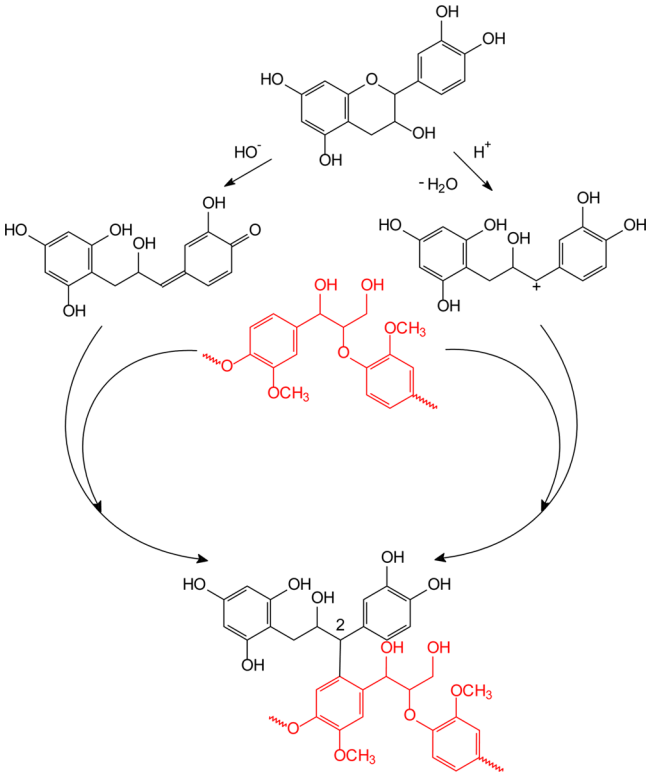

Figure 8. Schematic representation of lignin reaction with procyanidins under acidic and alkaline conditions.

basis of integral of signals at 59.0−61.0 ppm (C_γ resonance in *β*-*O*-4′ structures). The amounts of *β*-5′and *β*-*β*′ structures were assessed on the basis of C_β resonances in corresponding structures at 52.5−53.3 and 53.3−54.0 ppm, respectively, and the amount of *β*-1′ and *β*-5′ structures was calculated on the basis of the C_γ resonances in the spectrum region at 62.0−64.0 ppm (Table 3).

The data on abundance of *β*-*O*-4′ structures (Table 3) obtained by analysis of the ^{13}C NMR (0.39/C_6) in grape stalk dioxane lignin were consistent with those obtained by the ^{1}H NMR spectrum (0.38/C_9) (Table 3). A very similar abundance of *β*-*O*-4′ structures was reported previously for reed[14] and kenaf[13] dioxane lignins isolated from internode morphologic regions. ^{13}C NMR spectroscopy analysis (Table 3) also confirms a relatively high degree (Ar-C index of 1.50) of lignin condensation in grape stalks, which is comparable only to lignins isolated from nodes of reed.[14] According to the results of the PO analysis, most of the condensed lignin structures (ca. 17% mol) belongs to guaiacyl units substituted at C-5 and C-6 of the aromatic ring by aliphatic moieties (Figure 2). Meanwhile, the contribution of *β*-5′ structures (ca. 3% mol) (Table 3) to the total amounts of such condensed structures is not major. It may be proposed that a significant contribution to lignin condensation is due to its structural association with tannins. The condensation of tannins and lignin via ionic (e.g., quinone methide) mechanisms could occur under neutral/weakly acidic conditions due to tannin migration by diffusion from disintegrated cells to lignified cell tissues. Cell integrity may be violated both by natural reasons (e.g., down-regulated vacuole disrupts in parenchyma cells containing tannins) or by external factors during winemaking (e.g., during harvesting and destemming). In the last case the tannins from crushed grapes should also be taken into consideration. In addition, the contribution of the condensation reaction of lignin with tannins occurring under both alkaline (pre-extraction of grape stalks) and acidic (acidolysis) conditions during lignin isolation procedure cannot be excluded as well.

In conclusion, the results of the present study show that grape stalk lignin is of HGS type with a strong predominance of G units and a moderate amount of *β*-aryl ether structures, highly condensed, and, probably, structurally associated with tannins. This must be one of the reasons for difficult delignification of grape stalks by conventional pulping methods.

Unlike other lignins rich in G units, which possess a high abundance of biphenyl (5-5′), diaryl ether (4-*O*-5′), and *β*-5′structures, the high condensation degree of grape stalk lignin is due to unknown condensed structures of alkyl-aryl type. These structures, at least partially, must belong to lignin−tannin condensed structures. Among tannins involved in structural association with lignin, flavonols of gallocatechin and catechin types and ellagitannins were elucidated. The reasons for this lignin−tannin structural association are a challenge for future research.

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Notes

The authors declare no competing financial interest.

REFERENCES

(1) Meloni, G.; Swinnen, J. The political economy of European wine regulations. *J. Wine Econ.* **2013**, *8*, 244−284.

(2) Costa, J. M. Aproveitamento de subprodutos da vinificação. O bagaço como matéria-prima da indústria de óleos e grainha e de fabrico de rações para gado. In *First Congresso Nacional das Indústrias Agro-alimentares*; Junta Nacional dos Vinhos: Lisboa, Portugal, 1983.

(3) Spigno, G.; Pizzorno, T.; De Faveri, D. M. Cellulose and hemicelluloses recovery from grape stalks. *Bioresour. Technol.* **2008**, *99*, 4329−4337.

(4) Prozil, S. O.; Evtuguin, D. V.; Lopes, L. P. C. Chemical composition of grape stalks of *Vitis vinifera* L. from red grape pomaces. *Ind. Crops Prod.* **2012**, *35*, 178−184.

(5) Bertrán, E.; Sort, X.; Soliva, M.; Trillas, I. Composting winery waste: sludges and grape stalks. *Bioresour. Technol.* **2004**, *95* (2), 203−208.

(6) Bustamante, M. A.; Pérez-Murcia, M. D.; Paredes, C.; Moral, R.; Moreno-Caselles, J. Short-term carbon and nitrogen mineralisation in soil amended with winery and distillery organic wastes. *Bioresour. Technol.* **2007**, *98*, 3269−3277.

(7) Miralles, N.; Martınez, M.; Florido, A.; Casas, I.; Nuria Fiol, N.; Villaescusa, I. Grape stalks waste as low cost biosorbents: an alternative for metal removal from aqueous solutions. *Solv. Extr. Ion Exch.* **2008**, *26*, 261−270.

(8) Llobera, A.; Cañellas, J. Dietary fibre content and antioxidant activity of Manto Negro red grape (*Vitis vinifera*): pomace and stem. *Food Chem.* **2007**, *101*, 659−666.

(9) Cruz, J. M.; Dominguez, H.; Parajo, J. C. Assessment of the production of antioxidants from winemaking waste solids. *J. Agric. Food Chem.* **2004**, *52*, 5612−5620.

(10) Deiana, A. C.; Sardella, M. F.; Silva, H.; Amaya, A.; Tancredi, N. Use of grape stalk, a waste of the viticulture industry, to obtain activated carbon. *J. Hazard. Mater.* **2009**, *172*, 13−19.
(11) Ping, L.; Brosse, N.; Sannigrahi, P.; Ragauskas, A. Evaluation of grape stalks as a bioresource. *Ind. Crops Prod.* **2011**, *33*, 200−204.
(12) Jahan, M. S.; Chowdhury, D. A. N.; Islam, M. K.; Moeiz, S. M. I. Characterization of lignin isolated from some nonwood available in Bangladesh. *Bioresour. Technol.* **2007**, *98*, 465−469.
(13) Seca, A. M. L.; Cavaleiro, J. A. S.; Domingues, F. M. J.; Silvestre, A. J. D.; Evtuguin, D.; Pascoal Neto, C. Structural characterization of the bark and core lignins from kenaf (*Hibiscus cannabinus*). *J. Agric. Food Chem.* **1998**, *46*, 3100−3108.
(14) Seca, A. M. L.; Cavaleiro, J. A. S.; Domingues, F. M. J.; Silvestre, A. J. D.; Evtuguin, D.; Neto, C. P. Structural characterization of the lignin from the nodes and internodes of *Arundo donax* reed. *J. Agric. Food Chem.* **2000**, *48*, 817−824.
(15) Ping, L.; Brosse, N.; Chrusciel, L.; Navarrete, P.; Pizzi, A. Extraction of condensed tannins from grape pomace for use as wood adhesives. *Ind. Crops Prod.* **2011**, *33*, 253−257.
(16) Prozil, S. O.; Costa, E. V.; Evtuguin, D. V.; Lopes, L. P. C.; Domingues, M. R. M. Structural characterization of polysaccharides isolated from grape stalks of *Vitis vinifera* L. *Carbohydr. Res.* **2012**, *356*, 252−259.
(17) Souquet, J. M.; Labarbe, B.; Le Guernevé, C.; Cheynier, V.; Moutounet, M. Phenolic composition of grape stems. *J. Agric. Food Chem.* **2000**, *48*, 1076−1080.
(18) Ben-Ghedalia, D. Degradation of cell-wall monosaccharides of chemically treated grape branches by rumen microorganisms. *Eur. J. Appl. Microbiol. Biotechnol.* **1982**, *16*, 142−145.
(19) Neto, C. P.; Seca, A.; Fradinho, D.; Coimbra, M. A.; Domingues, F.; Evtuguin, D.; Silvestre, A.; Cavaleiro, J. A. S. Chemical composition and structural features of the macromolecular components of *Hibiscus cannabinus* grown in Portugal. *Ind. Crops Prod.* **1996**, *5*, 189−196.
(20) Evtuguin, D. V.; Neto, C. P.; Silva, A. M. S.; Domingues, P. M.; Amado, F. M. L.; Robert, D.; Faix, O. Comprehensive study on the chemical structure of dioxane lignin from plantation *Eucalyptus globulus* wood. *J. Agric. Food Chem.* **2001**, *49*, 4252−4261.
(21) Tanahashi, M.; Higuchi, T. Chemical degradation methods for characterization of lignins. In *Methods of Enzymology*; Abelson, J. N., Simon, M. I., Eds.; Academic Press: New York, 1988; pp 121−128.
(22) Gellerstedt, G. Chemical degradation methods: permanganate oxidation. In: *Methods in Lignin Chemistry*; Lin, S. Y., Dence, C. W., Eds.; Springer-Verlag: Berlin, Germany, 1992; pp 322−333.
(23) Zakis, G. F. *Functional Analysis of Lignins and Their Derivatives*; Tappi Press: Atlanta, GA, USA, 1994; pp 3−7.
(24) Evtuguin, D. V.; Domingues, P. N.; Amado, F. L.; Pascoal Neto, C.; Ferrer Correia, A. Electrospray ionization mass spectrometry as a tool for lignins molecular weight and structural characterization. *Holzforschung* **1999**, *53*, 525−528.
(25) Sears, K. D.; Casebier, R. L.; Hergert, H. L.; Stout, G. H.; McCandlish, L. E. The structure of catechinic acid. A base rearrangement product of catechin. *J. Org. Chem.* **1974**, *39*, 3244−3247.
(26) Pantoja-Castro, M. A.; González-Rodríguez, H. Study by infrared spectroscopy and thermogravimetric analysis of tannins and tannic acid. *Rev. Latinoam. Quim.* **2011**, *39*, 107−112.
(27) Faix, O. Fourier transform infrared spectroscopy. In *Methods in Lignin Chemistry*; Lin, S. Y., Dence, C. W., Eds.; Springer-Verlag: Berlin, Germany, 1992; pp 83−109.
(28) Herbert, H. L. Infrared spectra. In *Lignins. Occurrence, Formation, Structure and Reactions*; Sarkanen, K. V., Ludwig, C. H., Eds.; Wiley-Interscience: New York, 1971; pp 267−297.
(29) Oliveira, L.; Evtuguin, D. V.; Cordeiro, N.; Silvestre, A. J. D.; Silva, A. M. S.; Torres, I. C. Structural characterization of lignin from leaf sheaths of banana plant "Dwarf Cavendish". *J. Agric. Food Chem.* **2006**, *54*, 2598−2605.
(30) Oliveira, L.; Evtuguin, D.; Cordeiro, N.; Silvestre, A. J. D. Structural characterization of stalk lignin from banana plant. *Ind. Crops Prod.* **2009**, *29*, 86−95.
(31) Pinto, P. C.; Evtuguin, D. V.; Pascoal Neto, C. Chemical composition and structural features of the macromolecular components of plantation *Acacia mangium* wood. *J. Agric. Food Chem.* **2005**, *53*, 7856−7862.
(32) Fradinho, D. M.; Neto, C. P.; Evtuguin, D.; Jorge, F. C.; Irle, M. A.; Gil, M. H.; Pedrosa de Jesus, J. Chemical characterisation of bark and of alkaline bark extracts from maritime pine grown in Portugal. *Ind. Crops Prod.* **2002**, *16*, 23−32.
(33) Pinto, P. C.; Evtuguin, D. V.; Pascoal Neto, C. Effect of structural features of wood biopolymers on hardwood pulping and bleaching performance. *Ind. Eng. Chem. Res.* **2005**, *44*, 9777−9784.
(34) Robert, D. Carbon-13 Nuclear magnetic resonance spectrometry. In *Methods in Lignin Chemistry*; Lin, S. Y., Dence, C. W., Eds.; Springer Verlag: Berlin, Germany, 1992; pp 250−273.
(35) Kilpelaeinen, I.; Sipilae, J.; Brunow, G.; Lundquist, K.; Ede, R. M. Application of two-dimensional NMR spectroscopy to wood lignin structure determination and identification of some minor structural units of hard- and softwood lignins. *J. Agric. Food Chem.* **1994**, *42*, 2790−2794.
(36) Ralph, J.; Marita, J. M.; Ralph, S.; Hatfield, R. D.; Lu, F.; Ede, R. M.; Peng, J.; Quideau, S.; Helm, R. F.; Grabber, J. H.; Kim, H.; Jimenez-Monteon, G.; Zhang, Y.; Jung, H.-J. G.; Landucci, L. L.; MacKay, J. J.; Sederoff, R. R.; Chapple, C.; Boudet, A. Solution-state NMR of lignins. In *Advances in Lignocellulosics Characterization*; Argyropoulos, D. S., Ed.; Tappi Press: Atlanta, GA, USA, 1999; pp 55−108.
(37) Ralph, S. A.; Ralph, J.; Landucci, L. L. *NMR Database of Lignin and Cell Wall Model Compounds, 1996*; http://www.dfrc.wisc.edu/software.html (accessed March 22, 2006).
(38) Hise, R. G.; Chen, C. L.; Gratzl, J. S. Synthesis of β-aryl lignin model compounds. *J. Wood Chem. Technol.* **1985**, *5*, 379−390.
(39) Davis, A. L.; Cai, Y.; Davies, A. P.; Lewis, J. R. ^{1}H and ^{13}C NMR assignments of some green tea polyphenols. *Magn. Reson. Chem.* **1996**, *34*, 887−890.
(40) Porter, L. J.; Newman, R. H.; Foo, L. Y.; Wong, H.; Hemingway, R. W. Polymeric proanthocyanidins. ^{13}C N.M.R. studies of procyanidins. *J. Chem. Soc., Perkin Trans. 1* **1982**, *1*, 1217−1221.
(41) *Plant Polyphenols. Synthesis, Properties, Significance*; Hemingway, R. W., Laks, P. E., Eds.; Premium Press: New York, 1992; pp 295−338.
(42) Laks, P. E.; Hemingway, R. W.; Conner, A. H. Condensed tannins. Base-catalysed reactions of polymeric procyanidins with phloroglucinol: intramolecular rearrangements. *J. Chem. Soc., Perkin Trans. 1* **1987**, *6*, 1875−1881.
(43) Helm, R. F.; Ranatunga, T. D.; Chandra, M. Lignin-hydrolyzable tannin interactions in wood. *J. Agric. Food Chem.* **1997**, *45*, 3100−3106.
(44) Lange, W.; Faix, O. Lignin-polyphenol interaction in azobe (*Lophira alata*) heartwood. A study on milled wood lignin (MWL) and Klason residues. *Holzforschung* **1999**, *53*, 519−524.
(45) Rencoret, J.; Ralph, J.; Marques, G.; Gutiérrez, A.; Martínez, Á. T.; del Río, J. C. Structural characterization of lignin isolated from coconut (*Cocos nucifera*) coir fibers. *J. Agric. Food Chem.* **2013**, *61*, 2434−2445.
(46) 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*, 5922−5935.