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Isolation and Structural Characterization of Lignin from Cardoon (*Cynara cardunculus* L.) Stalks

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Abstract The lignin from *Cynara cardunculus* stalks was isolated by the classical Björkman method and characterized by pyrolysis coupled with gas chromatography and mass spectrometry (Py-GC/MS), two-dimensional nuclear magnetic resonance spectroscopy (2D-NMR), and derivatization followed by reductive cleavage (DFRC). The milled *Cynara* lignin (MCyL) was constituted mainly by guaiacyl (G) and syringyl-units(S) (S/G molar ratio of 0.7), with the complete absence of *p*-hydroxyphenyl (H) units. The 2D-NMR analysis indicated a predominance of alkyl-aryl ether linkages (70 % of all inter unit linkages are β–*O*–4′) and significant amounts of condensed structures such as phenylcoumarans (β-5′, 14 %), resinols (β-β′, 7 %), spirodienones (β-1′, 5 %), and dibenzodioxocins (5-5′, 4 %). Furthermore, the analyses indicated that the lignin is partially acylated at the γ-OH (12 % acylation) by acetate groups and that acetylation occurs preferentially on syringyl-units. As in other plants, acetylation occurs at the monomer stage, and sinapyl acetate behaves as a real lignin monomer participating in lignification in cardoon stalks. The detailed structural characterization of cardoon lignin reported here will foster the industrial use of this biomass for the production of biofuels and other bio-based chemicals under the lignocellulosic biorefinery.

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Keywords *Cynara cardunculus* · Milled *Cynara*lignin (MCyL) · Py-GC/MS · 2D-NMR · DFRC · S/G ratio

Introduction

Cardoon (*Cynara cardunculus* L.) is an herbaceous perennial crop from the Asteraceae family with high biomass productivity under the Mediterranean conditions [1–3].Cardoon has several industrial applications, the stalks can be used for energy and pulp and paper production, oil can be extracted from the seeds to produce biodiesel, polyphenols with pharmacological properties can be extracted from the leaves, andcardosins (milk protease) from the capitula are traditionally used for cheese production [1].

The cardoon stalks have already been chemically characterized and comprised 5 to 11 % ash, 13 to 21 % extractives, 13 to 23 % total lignin, and around 53 % polysaccharides [4–7]. Lignin is the second major component of the cell wall matrix in cardoon in amounts similar to what occurs in other herbaceous plants, such as wheat straw, corn, switch grass, or *Miscanthus*, where it can represent from 18 to 25 % [8].The lignin provides mechanical support for the plant, waterproofs the cell wall, enables the transport of water and nutrients, and provides a barrier against microorganisms. Some attempts have been made to isolate the lignin from woody and non-woody plants since it is a good raw material for the production of diverse useful products, e.g., phenol-formaldehyde resins [9] and bio-oils [10]. However, its use is still experimental or residual, mainly due to the difficulties for finding efficient, environmental, and economically viable solutions for its isolation from the lignocellulosic matrix [11]. For an appropriate valorization of cardoon as a raw material for the production of added-value products, the comprehensive characterization of their different components is of high interest, in particular

the composition and the structure of the lignin polymer, since it is a potential source of aromatic chemicals andbiofuels, as already mentioned.

However, studies regarding the detailed structure of the lignin of cardoon stalks are still scarce. Recently, a study was made to characterize the lignin in cardoon stalks by pyrolysis coupled with gas chromatography and mass spectrometry (Py-GC/MS), after fractionation in depithed stalks and pith [7]. The depithed stalks presented more lignin than pith (23.9 vs 21.8 %) and a syringyl to guaiacyl (S/G) ratio of 1.3 and 2.1, respectively. These previous studies were performed on cardoon stalks, and no efforts have been made so far to isolate its lignin for a detailed characterization or to evaluate its potential applications. To overcome this issue, in this paper, we report an exhaustive structural characterization of the lignin from cardoon stalks. For this purpose, we isolated the milled *Cynara* lignin (MCyL) according to the classical isolation protocol [12], which was subsequently analyzed by different analytical methodologies, including Py-GC/MS, two-dimensional nuclear magnetic resonance spectroscopy (2D-NMR), and derivatization followed by reductive cleavage (DFRC). Py-GC/MS is a reproducible and sensitive technique for the characterization of the composition of the lignin polymer, in terms of their *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units [13–15]. 2D-NMR complements the information obtained by pyrolysis and provides useful information regarding the lignin units and lignin interunit linkages [16, 17]. Finally, DFRC is a chemical degradative method that can give information on the occurrence of acylated γ-OH units. Altogether, these data will provide a detailed picture of the structure of cardoon lignin that will help to maximize the industrial exploitation of this plant as potential feedstock for the production of bio-based materials.

Material and Methods

Samples

Cardoon samples were collected in the final stage of their growth cycle, in an experimental field of Instituto Superior de Agronomia in Lisbon, Portugal. The stalks were separated from the leaves, cut in small pieces, and dried in an oven at 60 °C. The samples were milled in a knife mill (Retsch SM 2000), passing through a sieve of 6 mm×6 mm, and successively extracted with dichloromethane, ethanol, and water for 24 h each. The extracted samples were dried in an oven at 60 °C, milled in a knife mill (IKA MF10) passing through a 100-mesh sieve to obtain sawdust.

Chemical Analysis

Two samples were collected for the chemical characterization, and the following parameters were determined: ash content (TAPPI T211 om-02); total extractives determined from successive extraction in a Soxhlet apparatus with dichloromethane, ethanol, and water (TAPPI T204 cm-07); total lignin determined in the extracted material as the sum of Klason lignin (TAPPI T222 om-11); and acid-soluble lignin (UM 205 om-83). The neutral monosaccharide composition was determined in the hydrolysate from the lignin analysis and separated in an Aminotrap plus Carbopac SA10 column connected in a Dionex ICS-3000 High Pressure Ion Chromatography. The monosaccharides were reported as percentage of total monosaccharides.

Lignin Isolation

The milled *Cynara* lignin (MCyL) was prepared according to the classical procedure [12]. The sawdust was finely ball-milled in a Retsch PM100 planetarium ball mill at 400 rpm using a 500-mL agate jar and agate ball bearings (20×20 mm). The total ball-milling time for the samples was 5 h, with 5 min breaks after every 5 min of milling. The ball-milled powder (60.34 g) was extracted with dioxane-water (96:4, *v*/*v*) using 25 mL of solvent/g of milled sample, for 12 h under agitation. This solution was centrifuged, and the supernatant was evaporated to dryness at 40 °C at reduced pressure. The obtained residue, called raw MCyL (0.79 g), was dissolved into a solution of acetic acid/water (9:1, *v*/*v*) using 20 mL of solvent/g of raw MCyL. The lignin from the solution was precipitated into stirred cold water (225 mL/g of raw MCyL), and the formed precipitate was separated by centrifugation and milled in an agate mortar. This residue was dissolved in a solution of 1,2-dichcloroethane/ethanol solution (2:1, *v*/*v*) using 25 mL of this solution/g of lignin. After centrifugation to remove undissolved matter, the lignin in the supernatant was precipitated by adding the solution dropwise into diethyl ether, and the obtained residue was separated by centrifugation. The solid residue was suspended in diethyl ether overnight, centrifuged, and finally resuspended in petroleum ether overnight. The final purified MCyL sample was recovered by centrifugation and dried under N_2 current. The yield of the isolated MCyL preparation was approximately 10 % of the Klason lignin content.

Analytical Pyrolysis (Py-GC/MS)

The isolated MCyL (1.7 mg) sample was pyrolyzed in a EGA/Py-3030D microfurnace (Frontier Laboratories Ldt., Fukushima, Japan), connected to an Agilent 7820A GC system (Agilent Technologies, Inc., Santa Clara, CA) equipped with a DB-1701 fused silica capillary column (60 m×0.25 mm

Table 1 Chemical characterization of the whole stalks of *Cynara cardunculus* L.

	% o.d. mass
Ash	5.0
Total extractives	8.9
Dichloromethane	0.7
Ethanol	3.9
Water	4.3
Total lignin	19.2
Soluble lignin	2.1
Klason lignin	17.1
Monosaccharides (% of total neutral monosaccharides)	
Arabinose	0.8
Xylose	30.9
Mannose	1.2
Galactose	1.3
Glucose	65.8

i.d.×0.25 μm film thickness) and to a Agilent 5975 Mass detector (EI at 70 eV). The pyrolysis was performed at 500 °C during 1 min, and the interface was kept at 280 °C. The injector was at 250 °C, the oven temperature was programmed to start at 45 °C (4 min), increasing the temperature to 280 °C at a heating rate of 4 °C/min, and was maintained at 280 °C during 10 min. The carrier gas was helium with a flow of 2 mL min^{-1}. The compounds were identified by comparison of their mass spectra with those of the Wiley and NIST libraries and with those reported in the literature [18, 19] and, when possible, by comparison with the retention times and mass spectra of authentic standards. The peak molar areas of each compound were calculated, the summed areas were normalized and express as percentage.The response factors for most of the lignin-derived phenols released were practically identical, except for vanillin [20], which is a minor peak here. Therefore, no attempt was made to calculate the response factor for every single compound released.

Table 2 Identities and relative molar abundances (% of identified) of the lignin-derived compounds identified in the Py-GC/MS of the MCyL lignin preparation isolated from *Cynara cardunculus* L.

Peak	Compound	Mw	Origin	Relative abundance %
1	guaiacol	124	G	10.0
2	4-methylguaiacol	138	G	9.2
3	4-ethylguaiacol	152	G	2.3
4	4-vinylguaiacol	150	G	6.5
5	eugenol	164	G	1.5
6	4-propylguiacol	166	G	0.9
7	syringol	154	S	10.2
8	*cis*-isoeugenol	164	G	2.0
9	*trans*-isoeugenol	164	G	6.8
10	4-methylsyringol	168	S	7.6
11	vanillin	152	G	6.0
12	4-propinylguaiacol	162	G	0.5
13	4-propinylguaiacol	162	G	0.5
14	4-ethylsyringol	182	G	1.4
15	homovanillin	166	G	1.2
16	vanillic acid methyl ester	182	G	0.7
17	acetovanillone	166	G	3.1
18	4-vinylsyringol	180	S	4.0
19	4-propylsyringol	196	S	0.4
20	guaiacylacetone	180	G	0.6
21	4-allylsyringol	194	S	1.2
22	*cis*-4-propenylsyringol	194	S	1.9
23	4-propinylsyringol	192	S	0.3
24	4-propinylsyringol	192	S	0.2
25	*trans*-4-propenylsyringol	194	S	4.9
26	syringaldehyde	182	S	4.9
27	homosyringaldehyde	196	S	0.8
28	syringic acid methyl ester	212	S	0.6
29	acetosyringone	196	S	2.3
30	syringylacetone	210	S	0.4
31	*trans*-coniferaldehyde	178	G	4.2
32	propiosyringone	210	S	0.6
33	*trans*-sinapaldehyde	208	S	2.4
	S/G molar ratio			0.79

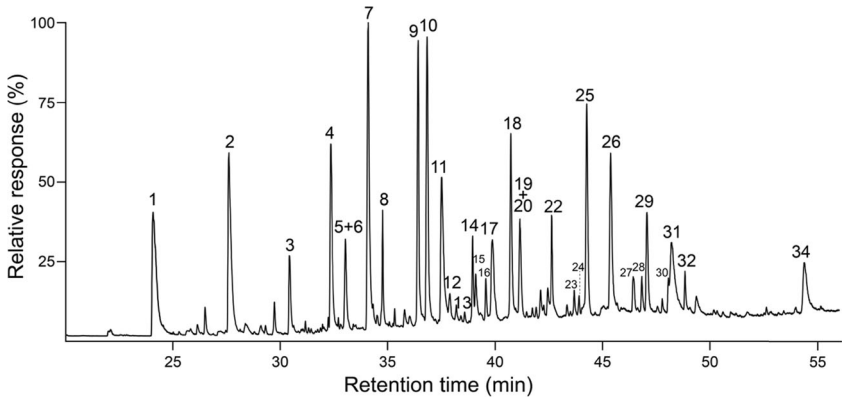

Fig. 1 Py-GC/MS chromatogram of the lignin isolated from *Cynara cardunculus* L. The identities and relative abundances of the released lignin-derived compounds are listed in Table 2

2D-NMR Spectroscopy

Around 30 mg of MCyL sample was dissolved in 0.75 mL of DMSO-d_6 for the NMR analysis. Heteronuclear single quantum correlation (HSQC) spectra were recorded at 300 K on a Bruker AVANCE III 500 MHz spectrometer (Bruker Biospin, Fallanden, Switzerland), equipped with a cryogenically cooled 5 mm TCI gradient probe with inverse geometry (proton coils closest to the sample). The 2D ^{13}C-^{1}H correlation spectra were obtained using an adiabatic HSQC pulse program (Bruker standard pulse sequence "hsqcetgpsisp2.2"). The spectral width was from 10 to 0 ppm (5000 Hz) in F_2 for ^{1}H dimension, with an acquisition time of 145 ms and a recycle delay (d1) of 1s. For the ^{13}C dimension, the spectral width was from 200 to 0 ppm (25,168 Hz) in F_1, being collected 256 increments of 32 scans for a total acquisition time of 2 h 40 min. The $^{1}J_{CH}$ used was 145 Hz. Processing used typical matched Gaussian apodization in ^{1}H and a squared cosine bell in ^{13}C. The central solvent peak was used as an internal reference (δ_C 39.5; δ_H 2.49 ppm).

2D-NMR HSQC cross-signals were assigned after comparison with data from literature [17, 21–25]. A semi-quantitative analysis of the volume integrals of the HSQC correlation peaks was performed using Bruker's Topspin 3.1 processing software. Integration of signals corresponding to chemically analogous C-H with similar $^{1}J_{CH}$ coupling values was performed separately for the different regions of the spectra. In the aliphatic oxygenated region, the relative abundances of side-chains involved in the various interunit linkages were estimated from the C_α–H_α correlations to avoid possible interference from homonuclear ^{1}H–^{1}H couplings, except for cinnamyl alcohol end-groups, for which C_γ–H_γ correlations had to be used. In the aromatic/unsaturated region, C_2–H_2 correlations from H, G, and S lignin units were used to estimate their relative abundances.

Derivatization Followed by Reductive Cleavage (DFRC)

To assess the incorporation of naturally γ-acetylated monolignols into the cardoon lignin, resulting in γ-acetylated lignin side-chains, a modification of the standard DFRC method using propionylating instead of acetylating reagents (so-called DFRC′) was used [26]. Lignin (5 mg) was stirred for 2 h at 50 °C with propionyl bromide in propionic acid (8:92, *v*/*v*). The solvents and excess bromide were removed by rotary evaporation. The products were then dissolved in dioxane/propionic acid/water (5:4:1, *v*/*v*/*v*), and 50 mg powdered Zn was added. After stirring for 40 min at room temperature, the mixture was transferred into a separatory funnel with dichloromethane and saturated ammonium chloride. The aqueous phase was adjusted to pH <3 by adding 3 % HCl, the mixture vigorously mixed, and the organic layer separated. The water phase was extracted twice more with dichloromethane. The combined dichloromethane fractions were dried over anhydrous $NaSO_4$, and the filtrate was evaporated to dryness using a rotary evaporator. The residue was subsequently propionylated for 1 h in 1.1 mL of dichloromethane containing 0.2 mL of propionic anhydride and 0.2 mL pyridine. The propionylated (and naturally acetylated) lignin degradation compounds were collected after rotary evaporation of the solvents, and subsequently analyzed

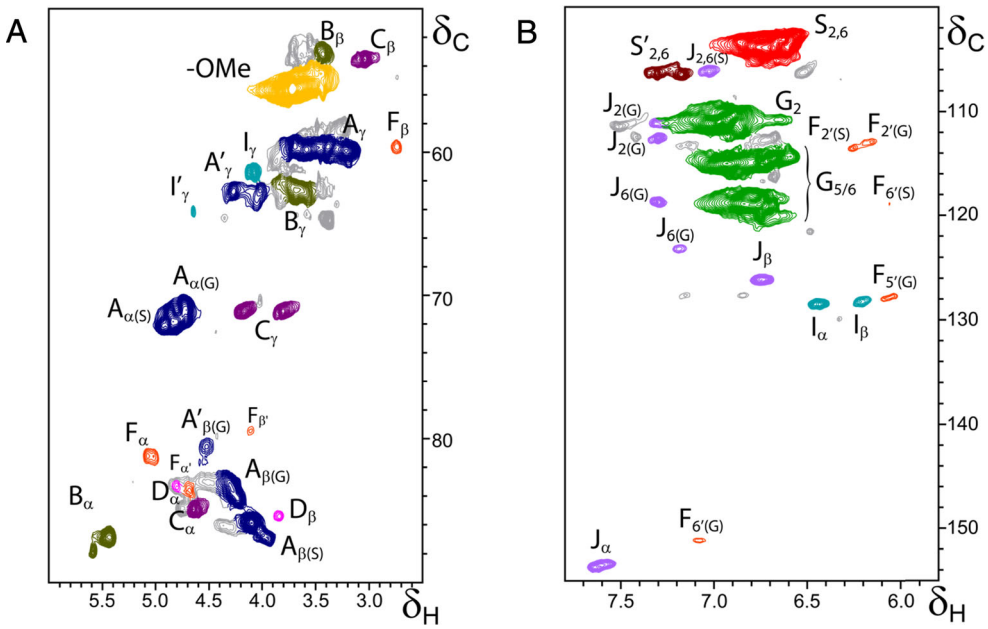

Fig. 2 **a** Side-chain and **b** aromatic/unsaturated regions in the HSQC NMR spectra of the lignin isolated from *Cynara cardunculus* L. The signal assignments are presented in Table 3, and the main lignin structures identified are depicted in Fig. 3

by GC/MS. The GC/MS analyses were performed with a GCMS-QP2010 Ultra instrument (Shimadzu Co.) using a capillary column (DB-5HT 30 m×0.25 mm I.D., 0.10 μm film thickness). The oven was heated from 140 °C (1 min) to 250 °C at 3 °C/min, then ramped at 10 °C/min to 300 °C, and held for 10 min at the final temperature. The injector was set at 250 °C, and the transfer line was kept at 300 °C. Helium was used as the carrier gas at a rate of 1 mL/min.

Results and Discussion

Chemical Characterization of Cardoon Stalks

The chemical characterization of the cardoon stalks is presented in Table 1. The stalks presented a high ash content of 5 %; similar and even higher ash contents have been reported in cardoon stalks by several authors [4, 5]. The extractives

Table 3 Assignments of the lignin ^{13}C–^{1}H correlation peaks in the 2D HSQC spectra of cardoon and the isolated MCyL

Label	δ_C/δ_H	Assignment
B_β	53.1/3.43	C_β–H_β in phenylcoumaran substructures (**B**)
C_β	53.5/3.05	C_β–H_β in β–β′ resinol substructures (**C**)
–OCH_3	55.6/3.73	C−H in methoxyls
A_γ	59.4/3.40 and 3.72	C_γ–H_γ in β–*O*–4′ substructures (**A**)
F_β	59.5/2.75	C_β–H_β in spirodienone substructures (**F**)
I_γ	61.3/4.08	C_γ–H_γ in cinnamyl alcohol end-groups (**I**)
B_γ	62.6/3.67	C_γ–H_γ in phenylcoumaran substructures (**B**)
A'_γ	63.5/3.83 and 4.30	C_γ–H_γ in γ-acylated β–*O*–4′ substructures (**A'**)
I'_γ	64.1/4.77	C_γ–H_γ in γ-*p*-coumaroylated cinnamyl alcohol end-groups (**I'**)
C_γ	71.0/3.83 and 4.19	C_γ–H_γ in β–β′ resinol substructures (**C**)
A_α/A'_α	71.1/4.71	C_α–H_α in β–*O*–4′ substructures (**A, A'**)
$F_{\beta'}$	79.4/4.10	$C_{\beta'}$–$H_{\beta'}$ in spirodienone substructures (**F**)
$A'_{\beta(G)}$	80.8/4.52	C_β–H_β in γ-acylated β–*O*–4′ substructures linked to a G-unit (**A'**)
F_α	81.2/5.01	C_α–H_α in spirodienone substructures (**F**)
D_α	83.0/4.82	C_α-H_α in 5-5' (dibenzodioxocin) substructures (**D**)
$F_{\alpha'}$	83.6/4.68	$C_{\alpha'}$–$H_{\alpha'}$ in spirodienone substructures (**F**)
$A_{\beta(G)}$	83.7/4.26	C_β–H_β in β–*O*–4′ substructures (**A**) linked to a G unit
C_α	84.7/4.64	C_α–H_α in β–β′ resinol substructures (**C**)
D_β	85.2/3.85	C_β–H_β in 5-5' (dibenzodioxocin) substructures (**D**)
$A_{\beta(S)}$	85.8/4.09	C_β–H_β in β–*O*–4′ substructures linked (**A**) to a S unit
B_α	86.8/5.43	C_α–H_α in phenylcoumaran substructures (**B**)
$S_{2,6}$	103.7/6.68	C_2–H_2 and C_6–H_6 in etherified syringyl units (**S**)
$J_{2,6(S)}$	106.2/7.02	C_2-H_2 and C_6-H_6 in sinapaldehyde end-groups (**J**)
$S'_{2,6}$	106.3/7.32 and 7.20	C_2-H_2 and C_6-H_6 in C_α-oxidized syringyl units (**S'**)
G_2	110.8/6.96	C_2–H_2 in guaiacyl units (**G**)
$J_{2(G)}$	111.2/7.31	C_2–H_2 and C_6-H_6 in coniferaldehyde end-groups (**J**)
$J_{2(G)}$	112.5/7.30	C_2–H_2 in coniferaldehyde end-groups (**J**)
$F_{2'(G)}$	113.0/6.17	$C_{2'}$–$H_{2'}$ in spirodienone substructures (**F**)
$F_{2'(S)}$	113.5/6.25	$C_{2'}$–$H_{2'}$ in spirodienone substructures (**F**)
G_5/G_6	115.0/6.74	C_5–H_5 and C_6–H_6 inguaiacyl units (**G**)
G_5	118.7/6.77	C_5–H_5 inguaiacyl units (**G**)
$J_{6(G)}$	118.8/7.30	C_6–H_6 in coniferaldehyde end-groups (**J**)
$F_{6'(S)}$	118.9/6.06	$C_{6'}$–$H_{6'}$ in spirodienone substructures (**F**)
$J_{6(G)}$	123.2/7.19	C_6–H_6 in coniferaldehyde end-groups (**J**)
J_β	126.3/6.76	C_β–H_β in cinnamaldehyde end-groups (**J**)
$F_{5'(G)}$	127.9/6.06	$C_{5'}$–$H_{5'}$ in spirodienone substructures (**F**)
I_β	128.4/6.23	C_β–H_β in cinnamyl alcohol end-groups (**I**)
I_α	128.4/6.44	C_α–H_α in cinnamyl alcohol end-groups (**I**)
$F_{6'(G)}$	151.2/7.07	$C_{6'}$–$H_{6'}$ in spirodienone substructures (**F**)
J_α	153.4/7.61	C_α–H_α in cinnamaldehyde end-groups (**J**)

accounted for 8.9 %, in the range of values already reported for this plant [4–6, 27]. Nevertheless, this value is slightly higher than that reported for other herbaceous plants, such as *Miscanthus* × *giganteus* (4.1 %) [28], but lower than the values reported for elephant grass (10.5 to 12.7 %) [22]. The high extractive content in cardoon stalks was mainly due to polar compounds (3.9 % of ethanol solubles and 4.3 % of water solubles), while the lipophilic compounds represented only a minor fraction (0.7 %), near to the range (1 to 2 %) previously reported [29].

The total lignin content in cardoon stalks was 19.2 %, a value slightly higher than the 17.0 % reported by Pereira et al. [5], or the 16.4 % reported by Ballesteros et al. [27]. However, this lignin content is lower than in other herbaceous plants, such as *Miscanthus* × *giganteus*, with a lignin content of 21.7 % [28], or elephant grass, with 20.5 % of lignin content [22].

Table 1 also presents the monosaccharide composition, where glucose and xylose are the main compounds, representing 65.8 and 30.9 % of total monosaccharides, as reported in the literature [5].

Lignin Composition as Observed by Py-GC/MS

The pyrogram of the isolated MCyL is presented in Fig. 1. The identities and relative molar abundances of the released compounds are listed in Table 2. Pyrolysis of MCyL released phenolic compounds derived from guaiacyl (G) and syringyl (S) lignin units, whereas compounds derived from *p*-hydroxyphenyl (H) lignin units were completely absent. The predominant lignin-derived phenolic compounds released were guaiacol (**1**), 4-methylguaiacol (**2**), 4-vinylguaiacol (**4**), syringol (**7**), *trans*-isoeugenol (**9**), 4-methylsyringol (**10**), vanillin (**11**), 4-vinylsyringol (**18**), trans-4-propenylsyringol (**25**), and syringaldehyde (**26**), among others. In general,a similar distribution of lignin-derived compounds was obtained by pyrolysis of whole cardoon stalks [7]. In the pyrogram of the

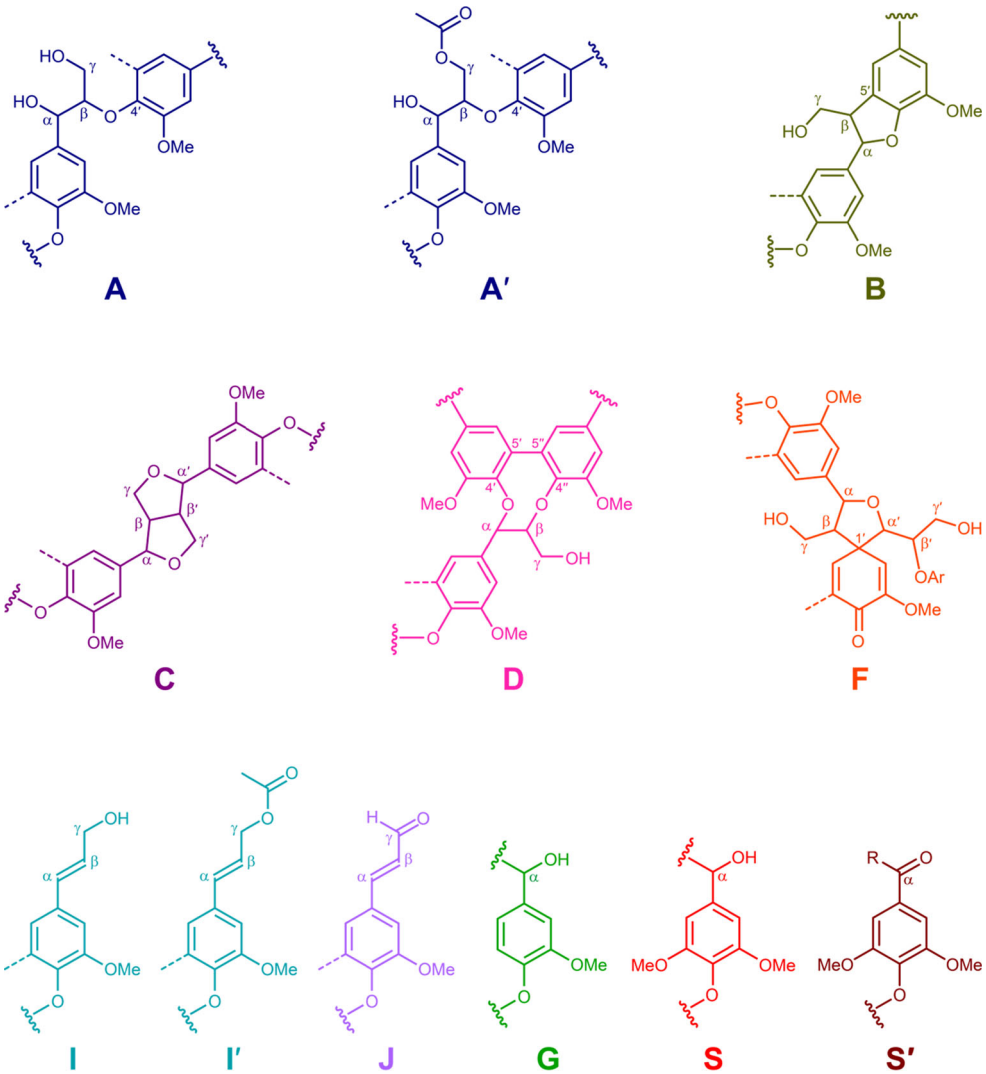

Fig. 3 Main structures present in the MCyL: **a** β-*O*-4′ alkyl-aryl ethers.**a′** β-*O*-4′ alkyl-aryl ethers with acylated γ-OH; **b** phenylcoumarans; **c** resinols; **d** dibenzodioxocins; **f** spirodienones; **i** cinnamyl alcohol end-groups; **j** cinnamaldehyde end-groups; **g** guaiacyl units; **s** syringyl units; **s** Cα-oxidized syringyl units

MCyL, the G-derived phenolic compounds were released in higher abundances than the respective S-lignin phenols, with a S/G molar ratio of 0.79.

The enrichment in G-lignin observed in cardoon stalks would make this lignin slightly recalcitrant toward depolymerization. This may affect the delignification stages during alkaline pulping, due to the lower reactivity of the G-lignin compared to S-lignin in alkaline systems [30]. The G units have a free C-5 position available for additional carbon-carbon or ether inter-unit bonds, which make them fairly resistant to lignin depolymerization during alkaline pulping. Therefore, lower S/G ratios imply lower delignification rates, more alkali consumption, and therefore lower pulp yield [14, 31].

Lignin Structural Units and Interunit Linkages as Seen by 2D-NMR

2D-NMR is a powerful technique for the elucidation of lignin structure and has been extensively applied for the characterization of lignocellulosic materials [21–23, 32]. The oxygenated aliphatic side-chain (δ_C/δ_H 50−90/2.5−6.0) and the aromatic (δ_C/δ_H 100−156/5.8−7.8) regions of the HSQC spectrum of the isolated MCyL are presented in Fig. 2. The main lignin cross-signals are assigned in Table 3, while the main lignin structures are depicted in Fig. 3.

The side-chain region of the HSQC spectrum (Fig. 2a) provides information of the interunit linkages in lignin. In this region of the spectrum cross-signals from methoxyls (δ_C/δ_H 55.6/3.73) and from β−*O*−4′ substructures (structure **A**) are the most prominent. Interestingly, the HSQC spectrum clearly showed the presence of intense signals in the range from δ_C/δ_H 63.5/3.83–4.30 and at 64.1/4.77 corresponding to the C_γ–H_γ correlations of γ-acylated units (structures **A'** and **I'**). The HSQC spectrum therefore indicates that this lignin is, at least partially, acylated at the γ-position of the lignin side-chain, as discussed below. Signals for condensed structures were also detected in the HSQC spectrum, although with lower intensities, including signals for phenylcoumarans (**B**), resinols (**C**), dibenzodioxocins (**D**), and spirodienones (**F**). Signals for cinnamyl alcohol end-groups (**I**) were also observed in this region of the spectrum.

The aromatic region of the spectra showed signals for G- and S-lignin units. The G-lignin units showed prominent signals for C_2–H_2 correlations at δ_C/δ_H 110.8/6.96 and for C_5–H_5 and C_6–H_6correlations (δ_C/δ_H 115.0/6.74 and 118.7/6.77). The S-lignin units showed a prominent signal corresponding to the $C_{2,6}$–$H_{2,6}$ correlations at a δ_C/δ_H 103.7/6.68 in etherified S-units ($\mathbf{S}_{2,6}$). Signals corresponding to $C_{2,6}$–$H_{2,6}$ correlations in Cα-oxidized S-lignin units ($\mathbf{S'}_{2,6}$) were observed at δ_C/δ_H 106.3/7.32 and 7.20. Signals for H-lignin units were not observed in the HSQC spectrum, in agreement with the results obtained by Py-GC/MS. In this region of the HSQC spectrum, it was also possible to detect signals from cinnamyl alcohol end-groups (**I**) and cinnamaldehyde end-groups (**J**). The total relative content of the cinnamaldehyde end-groups was estimated by comparison of the intensities of the C_β–H_β correlations in cinnamyl alcohols (**I**) and aldehydes (**J**). In addition, signals for the aromatic units of the spirodienones (**F**) are also observed in this region.

The relative abundances of the main inter-unit linkages (referred to as the total side-chains), cinnamyl end-groups, percentage of acylation of the lignin side-chain, as well as the relative abundances of the H-, G-, and S-units and the S/G ratio in MCyL, calculated from volume integrations in the HSQC spectrum, are shown in Table 4. The results indicate that the main lignin substructure present in MCyL is the β−*O*−4′ alkyl-aryl ether linkages (**A**), accounting for 70 % of all side-chains, followed by important amounts of phenylcoumarans (14 %), resinols (7 %), dibenzodioxocins (4 %), and spirodienones (5 %). Cinnamyl end-groups (**I, J**) were present in relatively high abundances, and accounting each one for 6 % of all inter-unit linkages. An estimation of the percentage of γ-acylation of the lignin side-chain in β−*O*−4′ structures was performed by integration of the signals corresponding to the hydroxylated versus acylated C_γ-H_γ correlations, and amounted to 12 % of the lignin side-chains. The same12 % value was also obtained for γ-acylated cinnamyl alcohol end-groups (**I'**) by integration of their signals. This is a rather low value when compared to those obtained in other plants such as sisal, elephant grass, or *Miscanthus* that presented, respectively, 68, 39–55, and 46 % of acylation [22, 33, 34]. Lignin acylation

Table 4 Structural characteristics (lignin interunit linkages, end-groups, percentage of γ-acetylation, aromatic units, and S/G ratio) from integration of ^{13}C-^{1}H correlation peaks in the HSQC spectrum of the isolated lignin (MCyL) from *Cynara cardunculus* L.

	MCyL
Lignin inter-unit linkages (%)	
β–*O*–4′ aryl ethers (**A/A'**)	70
Phenylcoumarans (**B**)	14
Resinols (**C**)	7
Dibenzodioxocins (**D**)	4
Spirodienones (**F**)	5
Lignin end-groups[a]	
Cinnamyl alcohol end-groups (**I**)	5
γ-acylated cinnamyl alcohol end-groups (**I'**)	1
Cinnamaldehyde end-groups (**J**)	6
Lignin side-chain γ-acetylation (%)	12
Lignin aromatic units[b]	
H (%)	0
G (%)	58
S (%)	42
S/G ratio	0.7

[a] Expressed as a fraction of the total lignin interunit linkage types **A**–**F**

[b] Molar percentages (H+G+S=100)

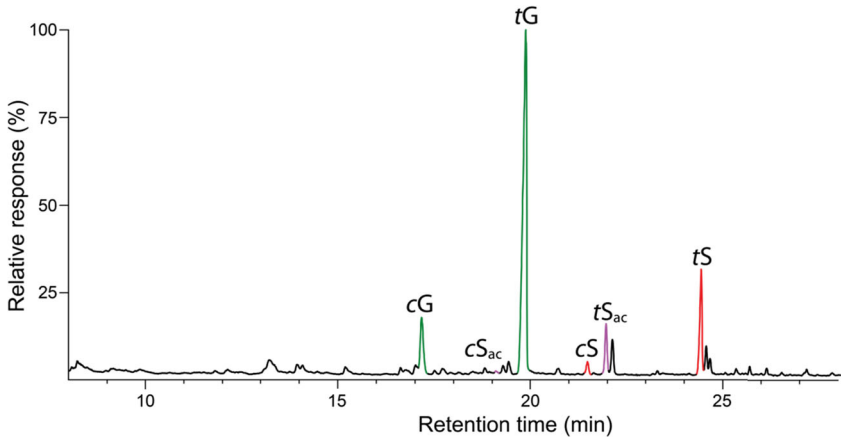

Fig. 4 Chromatogram (GC-TIC) of the DFRC′ degradation products from the MCyL isolated from *Cynara cardunculus* L. *c*G, *t*G, *c*S, and *t*S are the normal *cis*- and *trans*-coniferyl and sinapyl alcohol monomers (as their dipropionylated derivatives). *c*Sac and *t*Sac are the natively γ-acetylated *cis*- and *trans*-sinapyl alcohol (syringyl) monomers (as their phenolpropionylated derivatives)

in cardoon stalks occurred exclusively at the Cγ position, as also reported for other plants [35]. The S/G ratio estimated by NMR was 0.7 showing a slightly predominance of the G-lignin-derived phenols comparatively to S-lignin-derived phenols, as already observed by Py-GC/MS. The relatively high abundance of condensed lignin structures and the slight enrichment in G-lignin units would make the cardoon stalks more difficult to delignify than other herbaceous plants with higher S/G ratios and lower amounts of condensed linkages.

Lignin Acylation with Acetates as Seen by DFRC′ Analysis

The HSQC data shown above indicate that the lignin in MCyL is partially acylated at the γ-position of the side-chain, but cannot provide information on the nature of the acylating group. The DFRC degradation method, which cleaves α- and β-ether linkages in the lignin polymer leaving γ-esters intact [36, 37], seems to be the most appropriate method for the analysis of γ-acylated lignins. The DFRC degradation method originally developed does not allow the analysis of natively acetylated lignin units because the degradation products are acetylated during the procedure. However, with appropriate modification of the original protocol by replacing acetylating reagents with propionylating ones (so-called DFRC′), it is possible to obtain information about the presence of acetate groups originally acylating the γ-OH in lignin [26, 35, 38]. The chromatogram of the DFRC′ degradation productsof the MCyL is shown in Fig. 4. The released products are the *cis* and *trans* isomers of guaiacyl (*c*G, *t*G) and syringyl (*c*S, *t*S) lignin monomers (as their propionylated derivatives) arising from normal γ-OH units in lignin. In addition, the presence of originally γ-acetylated lignin units (cS_{ac} and tS_{ac}) were also detected in the chromatogram, and confirming that acetylation occurred exclusively at the γ-carbon of the lignin side-chain, as already observed in the HSQC spectra.The data also indicated that acetylation occurs preferentially over syringyl units (32 % of S-units and only 1 % of G-units are acetylated), as previously noted for most lignins [35, 38, 39]. Sinapyl acetate has been proposed to act as a real lignin monomer participating in lignification [35, 38, 40, 41]. The demonstration derived from the β-β′ coupling. If the γ-carbon of a monolignol is pre-acylated, the

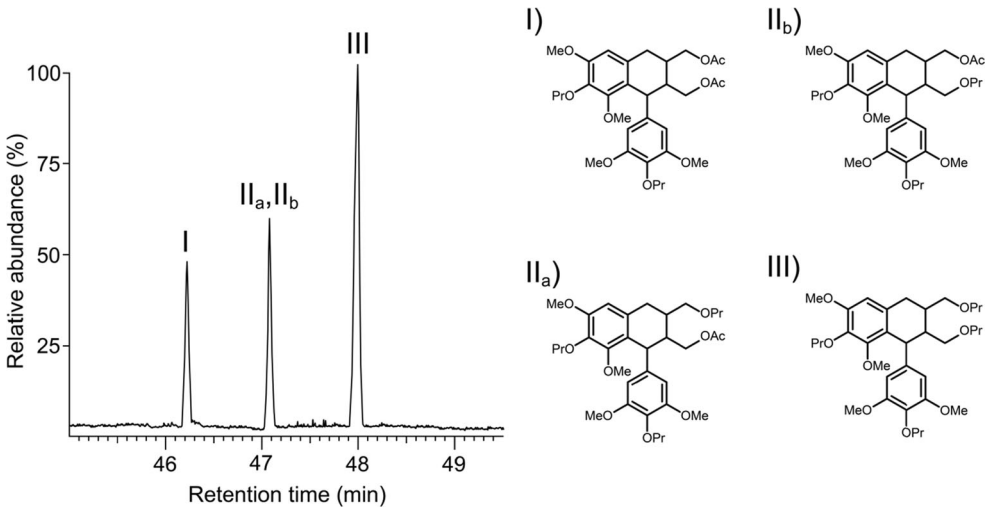

Fig. 5 Reconstructed ion chromatogram (sum of the ions at *m/z* 560, 574, and 588) and structures of the aryltetralin compounds arising from DFRC′ degradation of lignin β-β′ structures containing two (I), one (II_a and II_b), and none (III) native acetates and present in MCyL

formation of the normal β-β′ resinol structures cannot occur due to the absence of free γ-hydroxyls needed to re-aromatize the quinone methide moiety. Instead, new tetrahydrofuran structures are formed from the β-β′ homo- and cross-coupling of two sinapyl (acylated and non-acylated) monolignols [35, 38, 40, 41]. Figure 5 shows the reconstructed chromatograms (sum of the single ion chromatograms of the respective base peaks) [35, 38] of the aryltetralin DFRC′ degradation products expected from the resinol and tetrahydrofuran dimers arising from the β-β′ coupling of the sinapyl monolignols. Interestingly, compounds derived from the DFRC′ of homo-coupling of sinapyl acetate (**I**) and cross-coupling of sinapyl acetate and sinapyl alcohol (**II_a** and **II_b**) were clearly observed in the chromatogram of the MCyL, indicating that in cardoon, lignin sinapyl alcohol is pre-acetylated and behaves as a real monolignol participating in post-coupling reactions, as occurs in other plants.

Conclusions

The content and structure of cardoon lignin has been studied in detail. For this, the milled cardoon lignin (MCyL) was isolated according to the classical protocol and its structural features studied by different methodologies (Py-GC/MS, NMR, and DFRC′). The lignin was characterized as being slightly enriched in G-units (S/G ratio of 0.7–0.8). The main lignin substructure present includes β–*O*–4′ alkyl-aryl ethers (70 % of all interunit linkages), followed by phenylcoumarans (14 %) and minor amounts of resinols (7 %), dibenzodioxocins (4 %), and spirodienones (5 %). The data indicated that the lignin is partially acylated at the γ-OH (12 % acylation of all lignin side-chains) with acetate groups that are preferentially attached over syringyl units (32 % of S-units and only 1 % of G-units are acetylated at the γ-OH). The occurrence of structures arising from coupling and cross-coupling of sinapyl alcohol and sinapyl acetate confirms that sinapyl acetate acts as a real monolignol involved in lignification reactions in cardoon stalks, as also occurs in other plants. The detailed characterization of the cardoon lignin polymer reported in this study will foster the use of this material in lignocellulosic biorefineries for the production of biofuels and bio-based materials.

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