International Journal of Biological Macromolecules 261 (2024) 129694

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Tritordeum, a hybrid cereal with a highly tricin-enriched lignin

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ARTICLE INFO

Keywords:
Tricin
2D-NMR
Agricultural residue
Cinnamic acids
Waste valorization

ABSTRACT

The lignin from tritordeum straw, a hybrid cereal from crossbreeding of durum wheat and wild barley, was isolated and chemically characterized. Its composition and structure were studied by analytical pyrolysis (Py-GC/MS), nuclear magnetic resonance spectroscopy (NMR), Derivatization Followed by Reductive Cleavage (DFRC) method, and gel permeation chromatography (GPC). The data revealed an enrichment of guaiacyl (G) units (H:G:S of 3:61:36), which had a significant impact on the distribution of inter-unit linkages. The predominant linkages were the β–O–4′ alkyl-aryl ethers (78 % of all linkages), with substantial proportions of condensed linkages such as phenylcoumarans (11 %), resinols (4 %), spirodienones (4 %), and dibenzodioxocins (2 %). Moreover, DFRC revealed that tridordeum straw lignin was partly acylated at the γ-OH with both acetates and *p*-coumarates. Acetates were principally attached to G-units, whereas *p*-coumarates were predominantly attached to S-units. Furthermore, and more importantly, tritordeum lignin incorporates remarkable amounts of a valuable flavone, tricin, exceeding 30 g per kilogram of straw. Given the diverse industrial applications associated with this high-value molecule, tritordeum straw emerges as a promising and sustainable resource for its extraction.

1. Introduction

Over the course of the last century, cereal breeders dedicated their efforts to creating hybrids cereal varieties with superior agronomic capabilities, heightened phytochemical concentrations, and advanced technological attributes [1]. In line with these objectives, tritordeum was developed nearly 50 years ago by researchers at the Institute for Sustainable Agriculture in Córdoba, Spain. It involved the crossing of durum wheat (*Triticum turgidum* L. (Thell.) ssp. *durum*) and a wild barley species (*Hordeum chilense* Roem. & Schult.) [2–5], which gave rise to this hybrid cereal with exceptional processing characteristics like wheat (despite the lower gluten content) and an interesting nutritional profile similar to barley [6].

The harvesting of tritordeum produces significant quantities of agricultural residues, particularly the straw, which can represent up to 55 % of the total weight of the plant. As the demand for tritordeum in food processing increases yearly, substantial volumes of tritordeum straw are expected to become available as agricultural residue. This residue primarily consists of polysaccharides, specifically cellulose and hemicelluloses, along with lignin. Consequently, it represents a promising renewable resource within the context of lignocellulosic biorefineries, offering potential applications in the production of biomaterials, chemicals, and biofuels. However, industrial processing of these agricultural residues poses challenges due to their recalcitrant nature, mainly attributed to the presence of lignin. On the other hand, lignin, being an aromatic biomacromolecule, holds great appeal for biorefineries [7,8]. In addition, the lignin of grasses, including tritordeum, incorporates high-value molecules such as the flavone tricin alongside the typical phenylpropanoid lignin units (*p*-hydroxyphenyl, H; guaiacyl, G; and syringyl, S), as well as the *p*-hydroxycinnamic (*p*-coumaric and ferulic) acids [9–11]. Tricin is commonly found in the stems and leaves of grasses, including cereal crops, where it occurs as free tricin, tricin-glycosides, flavolignans, flavolignan glycosides as well as integrated into the lignin structure [12]. Interestingly, the content of tricin integrated into the stem lignin of several common cereal crops (maize, rice, wheat, and oat) has been shown to surpass that of free tricin [11]. Lignin-incorporated tricin can only be linked through 4–*O*–β linkages and is therefore found as an end-group within the lignin structure [10]. Consequently, this unique positioning offers the potential for the easy liberation of tricin through mild chemical reactions. Tricin is a highly valuable molecule that has garnered special attention due to its antioxidant, anticancer, antiviral, anti-inflammatory, and

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https://doi.org/10.1016/j.ijbiomac.2024.129694
Received 16 October 2023; Received in revised form 9 January 2024; Accepted 21 January 2024
Available online 27 January 2024

antihistaminic activities, among others [13–15]. Given the significant content of tricin in the lignins of its wheat and barley progenitors [11], tritordeum is expected to contain noteworthy amounts of lignin-incorporated tricin. In this context, tritordeum straw emerges as a promising renewable resource to produce bioethanol, bioproducts, and high-value compounds like tricin.

The main objective of this work is to study, for the first time, the detailed composition and structure of lignin present in tritordeum straw. To achieve this objective, the current study encompassed the isolation of the lignin in native-like form, followed by an extensive analysis employing cutting-edge analytical techniques, including analytical pyrolysis (Py-GC/MS), nuclear magnetic resonance (^{31}P, 2D-HSQC, and 2D-HMBC), wet chemistry (Derivatization Followed by Reductive Cleavage, DFRC method), and gel permeation chromatography (GPC). This information will be essential for optimizing the utilization of this agricultural residue and boost the economy surrounding this remarkable hybrid crop.

2. Materials and methods

2.1. Tritordeum straw compositional analysis

Tritordeum plants were cultivated in Córdoba (Spain) during 2022. After harvesting, the straw was air-dried and knife-milled. To determine the plant cell wall non-polymeric components, a series of consecutive extractions were performed using acetone (8 h), methanol (8 h), and water (8 h) in a Soxhlet apparatus. Following each extraction step, the extracts were subjected to drying via a rotary evaporator, and their contents were assessed through gravimetric analysis. The total lignin content was calculated by combining Klason and acid-soluble lignins, which were quantified in accordance with the Tappi T222 om-88 and UM 250 methods, respectively [16]. The Klason lignin measurement was adjusted to account for the presence of protein and ash. The percentage of protein in tritordeum straw was calculated based on the nitrogen content, which was analyzed using an elemental analyzer (LECO CHNS-932), with a conversion factor of 6.25 [17], while the ashes, the residue that remained after incineration of tritordeum straw (600 °C, 6 h), was determined gravimetrically. The acid chlorite method was employed to measure the holocellulose (cellulose + hemicelluloses) content [18]. Following that, cellulose content was ascertained by selectively eliminating hemicelluloses from the holocellulose through alkali extraction [18]. Each determination was conducted in duplicate to ensure accuracy.

2.2. Lignin isolation

Lignin extraction from tritordeum straw followed the Björkman's method [19], which uses neutral solvents (dioxane:water) to yield a preparation widely recognized as a faithful representation of lignin in its natural form, known as 'milled-wood' lignin (MWL) [20,21]. To do so, extractives-free tritordeum straw underwent a grinding process in a ball mill (Retsch PM 100) for a duration of 5 h at 400 rpm, employing a 500 mL agate jar and agate ball bearings (20 × 20 mm). Subsequently, it was subjected to dioxane-water (96:4, v/v) extraction and further purification according to experimental conditions previously reported [22], detailed in the supplementary information (SI). The MWL yield amounted to ~15 % relative to the total lignin content of the initial material.

2.3. Analytical pyrolysis

The MWL isolated from tritordeum straw (~0.5 mg) was pyrolyzed in a Frontier 3030 micro-furnace pyrolyzer. The released phenolic compounds were separated on an Agilent 7820A GC equipped with a DB-1701 column and detected with a Agilent 5975 mass detector, using previously described experimental conditions [23]. To conduct pyrolysis in the presence of tetramethylammonium hydroxide (TMAH), approximately 0.5 mg of MWL was combined with 10 μL of TMAH (25 % w/w, in methanol) and the identical experimental analysis condition were applied as those used for the sample without TMAH. The identification of pyrolysis products was conducted by comparing their mass spectra with those described in the literature [24]. To determine the relative percentages of the phenolic compounds released from lignin, calculations were based on the molar peak areas, as previously described [23]. Detailed experimental conditions are included in the SI.

2.4. Chemical degradation of tritordeum straw lignin (DFRC method)

The chemical degradation of tritordeum straw MWL was achieved following the originally developed DFRC method [25,26], with some slight modifications detailed elsewhere [22]. The product released upon DFRC were identified and quantified through GC/MS analysis, employing the instrumentation and experimental parameters outlined in the SI.

2.5. 2D-NMR analyses

Around 40 mg of tritordeum straw lignin was introduced into an NMR tube and fully dissolved in 0.5 mL of deuterated dimethyl sulfoxide. NMR spectra (HSQC and HMBC) were acquired on a Bruker Avance III 500 MHz spectrometer equipped with a TCI 5 mm cryoprobe. Detailed information on the acquisition parameters, pulse programs, and processing of these spectra can be found in the SI. Assignation of the HSQC correlation peaks was carried out according to the literature [9,27,28], and the quantitation of linkages and lignin units was performed as described before [9,28], using Bruker's Topspin 4.2 software [29].

2.6. ^{31}P NMR analyses

To perform quantitative ^{31}P NMR analysis, the tritordeum straw MWL was subjected to duplicate analysis using a conventional phosphitylation procedure [30]. Briefly, approximately 20 mg of moisture-free MWL, meticulously weighed, was introduced into an NMR tube and dissolved in 300 μL of a pyridine/$CDCl_3$ solution (1.6/1.0, v/v). To this mixture were added 150 μL of an internal standard solution (*N*-hydroxy-5-norbornene-2,3-dicarboximide, NHND, 9.8 mg/mL) and 75 μL of a relaxation reagent solution (chromium (III) acetylacetonate, 10.5 mg/mL), both prepared independently using the aforementioned pyridine/$CDCl_3$ solution. Afterward, 75 μL of derivatization reagent, 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (TMDP), were added and the tube was shaken until a homogeneous solution was formed, which was immediately analyzed. The acquisition of the ^{31}P NMR spectra was performed on a Bruker Avance NEO 500 MHz equipment using the pulse program "zgig", a relaxation delay of 5 s and 256 accumulated scans. Chemical shifts were calibrated with respect to the distinct signal arising from the hydroxylated-TMDP, which appeared at 132.2 ppm. The assignments of the signals in the ^{31}P NMR spectrum were in accordance with the literature [30–32]. The quantification of hydroxyl groups was conducted by measuring the amount of NHND added (with a purity of 97 %). All solvents and reagents for the ^{31}P NMR analysis were purchased from Sigma Aldrich, except for NHND, which was acquired from Alfa Aesar. Peak integration was employed to estimate the quantities of the different OH-groups present in tritordeum lignin, using the following formula:

$$X = \frac{V \cdot A \cdot IS \cdot P}{M \cdot N}$$

where X = mmol of OH-groups per gram of lignin (mmol/g), V = volume of internal standard added (mL), A = peak area, IS = internal standard concentration (mg/mL), P = internal standard purity (0.97), M =

amount of lignin (g) and N = internal standard molecular mass (g/mol).

2.7. Lignin molecular weight determination

The molecular weight of tritordeum straw MWL was assessed by gel permeation chromatography (GPC). Before the analysis, the sample underwent acetylation (Ac_2O:Py, 1:1 v/v), and then dissolved in tetrahydrofuran (THF). The analysis was performed on a Shimadzu Prominence-i LC-2030 3D GPC system equipped with a PLgel 5 μm MIXED-D, 7.5 × 300 mm column. For in-depth experimental conditions, please refer to the SI.

3. Results and discussion

3.1. Main constituents of tritordeum straw

Before proceeding with the isolation and characterization of the lignin fraction, an initial analysis was conducted to determine the content of the main components of tritordeum straw (Table 1). The analysis revealed that tritordeum straw was primarily composed of polysaccharides (cellulose and hemicelluloses), accounting for 59.1 % of the total dry weight. Among these polysaccharides, cellulose constituted the major part, representing up to 40.2 % of the total dry weight, while hemicelluloses were present in a lower proportion (18.9 %). Lignin constituted the second most prevalent element in the cell wall of tritordeum straw, representing approximately 14.7 % of the total dry weight, encompassing both acid-insoluble (Klason lignin) (13.1 %) and acid-soluble (1.6 %) lignins. The protein content was substantial, representing up to 6.6 % of the total dry weight, significantly higher than the protein contents found in its wheat and barley parent crops, around 4 % and 5 % on a dry matter basis, respectively [33]. Methanol and hot-water extractives were also notable components, constituting approximately 6.9 % and 8.8 % respectively, while acetone extractives were present in smaller amounts (2.1 %). Finally, the ash content amounted to 3.4 %, based on dry mass.

Following a thorough analysis of the overall composition of tritordeum straw, the subsequent step involved the extraction of its lignin using neutral solvents [19]. This method is well-known for its use of mild extraction conditions, resulting in a lignin preparation similar to that found naturally within the cell wall [20,21].

3.2. Structural features of tritordeum straw lignin

3.2.1. Tritordeum straw lignin composition as determined by analytical pyrolysis

The composition of tritordeum straw MWL was initially examined using Py-GC/MS (Fig. 1A). Pyrolysis of tritordeum straw MWL released phenolic compounds derived from the *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) lignin units, as well as from *p*-hydroxycinnamates (*p*-coumarates, *p*CA, and ferulates, FA). The main phenolic compounds released included guaiacol (2), 4-methylguaiacol (5), 4-vinylphenol (8), 4-vinylguaiacol (9), syringol (13), *trans*-isoeugenol (17), 4-methylsyringol (18), vanillin (19), acetosyringone (34), and *trans*-coniferyl alcohol (35), among others. Table 2 provides a comprehensive account of the identities and relative abundances of the phenolic compounds released during pyrolysis.

Table 1
Abundance (%) of the main constituents of tritordeum straw[a].

Total extractives	**17.8 ± 1.8**
Acetone extractives	2.1 ± 0.3
Methanol extractives	6.9 ± 0.0
Hot-water extractives	8.8 ± 1.5
Total lignin content	**14.7 ± 0.2**
Klason lignin	13.1 ± 0.1
Acid-soluble lignin	1.6 ± 0.1
Holocellulose	**59.1 ± 0.2**
Hemicelluloses	18.9 ± 0.1
α-Cellulose	40.2 ± 0.1
Proteins	**6.6 ± 0.1**
Ashes	**3.4 ± 0.1**

[a] Average of two replicates and expressed as percentage of dry weight.

In theory, the H:G:S lignin composition can be estimated by examining the relative proportions of the different phenolic compounds originating from H, G, and S-lignin. Nevertheless, grasses also include *p*CA and FA in their lignins [9,23,29,34,35], which undergo decarboxylation upon pyrolysis producing 4-vinylphenol and 4-vinylguaiacol, respectively, that cannot be distinguished from those arising from H- and G-lignin units, and which severely hinder the estimation of H:G:S composition [36].

The occurrence of *p*CA and FA in tritordeum straw MWL was proved through pyrolysis conducted in the presence of TMAH. This chemical methylates phenolic and carboxyl groups, effectively inhibiting decarboxylation reactions [36,37]. The Py-TMAH-GC/MS chromatogram (Fig. 1B) revealed the substantial release of *p*CA and FA (detected as their fully methylated derivatives) from tritrodeum MWL, which is a common characteristic of lignin in grasses [9,23,29,34,35]. This indicates that a part of the 4-vinylphenol and 4-vinylguaiacol released upon pyrolysis can be attributed to the decarboxylation of *p*CA and FA. Consequently, these compounds cannot be relied upon to estimate the H:G:S lignin composition. In the context of approximating the H:G:S composition of lignin, it was prudent to exclude 4-vinylphenol and 4-vinylguaiacol (along with their respective 4-vinylsyringol counterparts), as has been previously practiced for various grass species [9,22,23,28,38]. The lignin composition thus estimated by analytical pyrolysis (Table 2) revealed that tritordeum straw lignin exhibited a substantial enrichment of G-lignin units at 57.5 %, followed by S-units at 32.8 %, with a lower proportion of H-units (9.7 %). The S/G ratio was calculated at 0.6, making it comparable in composition to that of wheat straw lignin [9].

3.2.2. Aromatic lignin units and main bonds as observed by NMR

Further insight into the structural attributes of tritordeum straw lignin, encompassing its aromatic units and primary interunit linkages, was garnered through 2D-NMR HSQC analysis (Fig. 2). The precise chemical shifts of the assigned HSQC signals are provided in Table S1. In the aliphatic-oxygenated region (Fig. 2A), distinct correlation signals were observed, each corresponding to different types of linkages present in the lignin structure. Within the aliphatic-oxygenated region, the most prominent correlation signals were attributed to the β−*O*−4′ linkages (A), with subsequent signals corresponding to phenylcoumarans (B), resinols (C), dibenzodioxocins (D), spirodienones (F), and cinnamyl alcohol end-groups (I). Other signals identified in the aliphatic-oxygenated region corresponded to γ-acylated β−*O*−4′ (A′), phenylcoumarans (B′), tetrahydrofurans (C′), and cinnamyl alcohol end-groups (I′) substructures. Interestingly, the signals observed at δ_C/δ_H 63.9/4.77 and 64.1/4.63 are characteristic of γ-acylated cinnamyl alcohol end-groups (I'_γ) esterified with *p*CA and acetates (Ac), respectively, indicating that these specific moieties function as the γ-acylating components within the structure of tritordeum straw lignin. Within the HSQC aromatic region (Fig. 2B), the most prominent signals were attributed to G- and S-lignin units, as well as tricin (T). Additionally, the spectrum also revealed signals originating from H-lignin units, ferulic acid (FA), *p*CA, as well as from cinnamaldehyde (J) and cinnamyl alcohol end groups (I).

From the 2D-HSQC-NMR spectrum, we obtained invaluable insights into the structural attributes of lignin in tritordeum straw. This includes assessments of the relative abundances of primary lignin inter-unit linkages, lignin end-groups, the extent of γ-acylation, the composition of lignin aromatic units, as well as the presence of tricin, *p*CA, and FA. The comprehensive data can be found in Table 3. The HSQC analysis

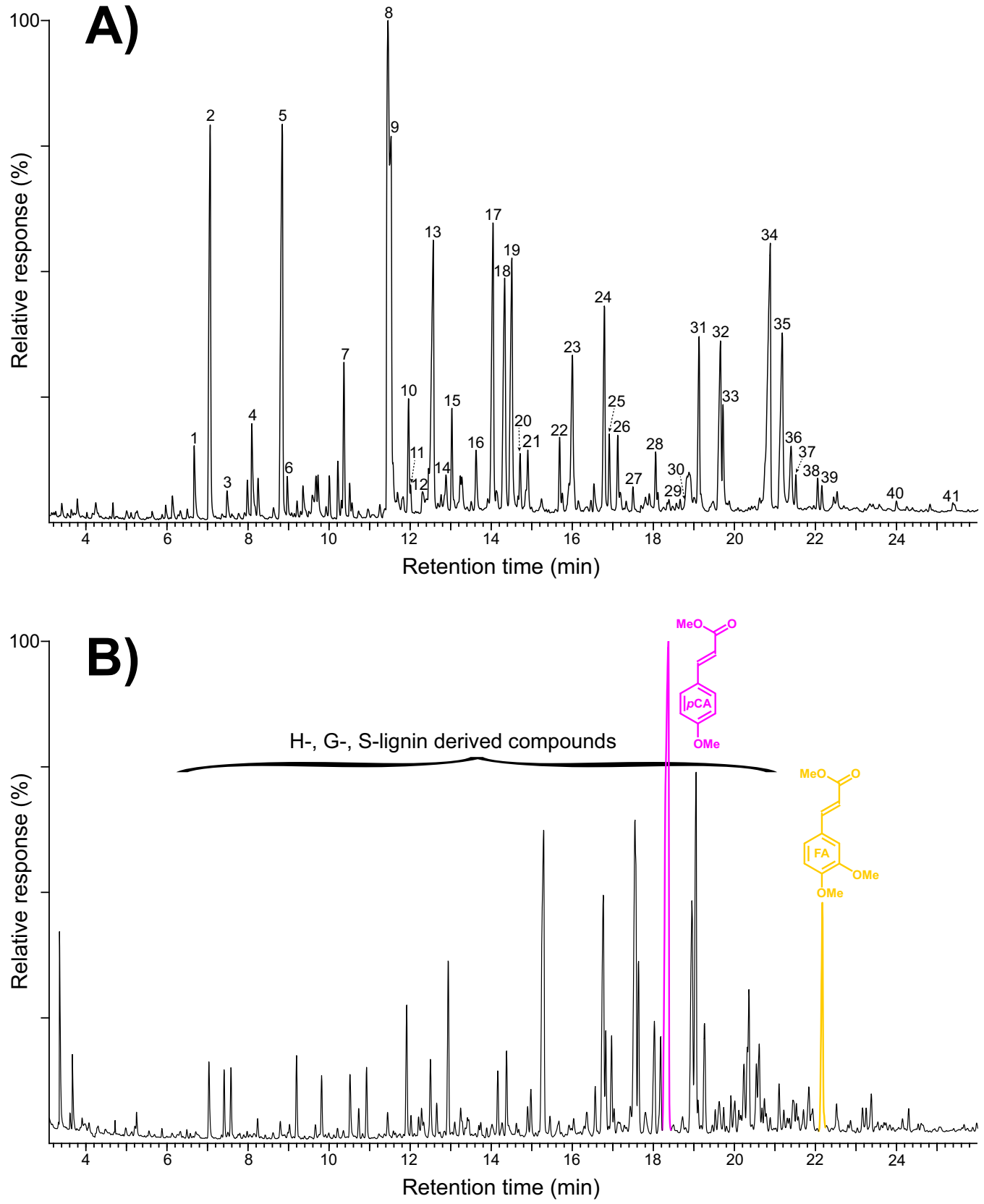

Fig. 1. Py-GC/MS (**A**) and Py-TMAH-GC/MS (**B**) chromatograms of the MWL isolated from tritordeum straw. The identities and relative abundances of the phenolic compounds released upon regular pyrolysis (in the absence of TMAH) are detailed in Table 2. In the Py-TMAH-GC/MS chromatogram, *p*CA is the fully methylated *p*-coumaric acid (methyl *trans*-4-*O*-methyl-*p*-coumarate) and FA is the fully methylated ferulic acid (methyl *trans*-4-*O*-methyl-ferulate).

Table 2
Identities and relative molar abundances of the main phenolic compounds identified among the pyrolysis products of tritordeum straw MWL.

N°	Compound	Origin[#]	Percentage (%)
1	phenol	H	1.6
2	guaiacol	G	7.9
3	3-methylphenol	H	0.6
4	4-methylphenol	H	2.0
5	4-methylguaiacol	G	8.3
6	4-ethylphenol	H	0.8
7	4-ethylguaiacol	G	1.9
8	4-vinylphenol	H/*p*CA	11.7
9	4-vinylguaiacol	G/FA	8.3
10	eugenol	G	1.5
11	4-propylguaiacol	G	0.4
12	4-allylphenol	H	0.7
13	syringol	S	6.1
14	*cis*-4-propenylphenol	H	0.4
15	*cis*-isoeugenol	G	1.5
16	*trans*-4-propenylphenol	H	1.4
17	*trans*-isoeugenol	G	5.2
18	4-methylsyringol	S	4.6
19	vanillin	G	5.3
20	propyne-guaiacol	G	0.7
21	propyne-guaiacol	G	0.9
22	4-ethylsyringol	S	0.8
23	acetovanillone	G	3.0
24	4-vinylsyringol	S	2.9
25	guaiacylacetone	G	1.0
26	4-allylsyringol	S	0.8
27	propiovanillone	G	0.4
28	*cis*-4-propenylsyringol	S	0.6
29	propyne-syringol	S	0.1
30	propyne-syringol	S	0.1
31	*trans*-4-propenylsyringol	S	2.3
32	syringaldehyde	S	2.7
33	*cis*-coniferyl alcohol	G	1.3
34	acetosyringone	S	5.9
35	*trans*-coniferyl alcohol	G	3.7
36	*trans*-coniferaldehyde	G	1.2
37	syringylacetone	S	0.3
38	propiosyringone	S	0.3
39	syringyl vinyl ketone	S	0.2
40	*cis*-sinapyl alcohol	S	0.1
41	*trans*-sinapyl alcohol	S	0.1
H (%)[a]			9.7
G (%)[a]			57.5
S (%)[a]			32.8
S/G ratio			0.6

#Abbreviations: H, *p*-hydroxyphenyl units; G, guaiacyl units; S, syringyl units; *p*CA, *p*-coumaric acid; FA, ferulic acid.
[a] Calculated without using 4-vinylphenol (8), 4-vinylguaiacol (9) and 4-vinylsyringol (24).

revealed that tritordeum straw lignin was mostly composed of G- (61 %) and S-units (36 %), with a smaller proportion of H-units (3 %). This resulted in an S/G ratio of 0.6, consistent with the Py-GC/MS data. The lignin also contained minor quantities of *p*-hydroxycinnamates, corroborating the Py-TMAH data. Specifically, *p*CA was detected at approximately 6 % of the total lignin content, while FA was found to be present at just 1 %. Even more noteworthy was the substantial presence of tricin integrated into this lignin, constituting roughly 24 % of the overall lignin content (where H + G + S collectively sums up to 100).

The increased prevalence of G-units in tritordeum straw lignin significantly influenced the distribution of various inter-unit linkages. The predominant linkages observed were the β–*O*–4′ alkyl–aryl ethers, constituting a substantial 78 % of the total linkages. Additionally, this lignin exhibited noteworthy levels of condensed linkages, including phenylcoumarans (11 %), resinols (4 %), spirodienones (4 %), along with minor occurrences of dibenzodioxocins (2 %). Finally, cinnamyl alcohol end-groups accounted for as much as 7 % concerning the total inter-unit linkages, while cinnamaldehyde end-groups represented only 2 %.

The HSQC analysis also indicated that the side-chains of tritordeum straw lignin were partly acylated (14 %) at the γ-OH, by *p*CA and Ac, as shown above. The nature of these γ-acylating groups in tritordeum straw lignin was further confirmed by 2D-HMBC NMR (Fig. 3), which displays long-range correlations (at 2–3 chemical bonds) between protons and carbons. The HMBC spectrum revealed the presence of two distinctive carbonyl carbon correlations, one at δ_C 165.9 (attributed to *p*CA) and another at δ_C 170.0 (associated with Ac). The confirmation of these assignments was strengthened by HMBC correlations between the carbonyl carbon at δ_C 165.9 and the H_7 and H_8 protons of *p*CA, which appeared at $\delta_H \sim 7.40$ and 6.30, respectively. Similarly, correlations between the carbonyl carbon at δ_C 170.0 and the methyl group protons of Ac, approximately in the range of $\delta_H \sim 1.9$–2.0 (details not shown), provided unequivocal validation of the assignments. The correlations of these two carbonyl carbons with protons in the $\delta_H \sim 4.0$–5.0 range constituted compelling evidence that *p*CA and Ac are indeed the acylating groups present in the lignin of tritordeum straw.

3.2.3. Lignin acylation as determined by DFRC

To gain further insights into the lignin side-chains γ-acylation, we subjected the tritordeum straw MWL to DFRC analysis, a chemical degradative technique specifically designed to break β-ether linkages within lignin while preserving the ester linkages that acylate the γ-OH. The DFRC chromatogram of tritordeum straw MWL (Fig. 4**)** displayed the *cis*- and *trans*-isomers of the three lignin units (*c*H and *t*H; *c*G and *t*G; *c*S and *t*S), as their acetylated monomers, arising from *γ*-OH (as well as from native *γ*-acetates, as will be shown below) β-ethers in the lignin. In addition, small peaks of γ-*p*-coumaroylated S-units (cS_{pCA} and tS_{pCA}) were detected, while there was no evidence of γ-*p*-coumaroylated G-lignin units, in agreement with the low amounts of *p*CA present in this lignin (6 % as shown by 2D-NMR). The presence of a remarkable tricin peak in the chromatogram aligns with the considerable amounts of tricin determined by 2D-HSQC-NMR. Additionally, since tricin can solely form 4′–*O*–β ether bonds [10], the ones cleaved upon DFRC, it can be assumed that all tricin molecules were liberated and detected among the DFRC products.

As previously mentioned, the HSQC and HMBC spectra provided evidence of γ-acylation with acetates in tritordeum straw lignin. However, the analysis of naturally acetylated lignin units using the original DFRC procedure is not possible due to the acetylation that the degradation products undergo during the protocol. To overcome this limitation, we employed an adapted variant of the method known as DFRC' [26], wherein the acetylating agents were substituted with propionylating reagents. This modification allowed us to obtain insights into native acetylated lignin units. Fig. 5 displays the chromatogram of the degradation products liberated from tritordeum straw MWL using the DFRC' method. Intriguingly, DFRC' effectively released substantial quantities of γ-acetylated *cis*- and *trans*-isomers of lignin monomers (tH_{ac}; cG_{ac} and tG_{ac}; cS_{ac} and tS_{ac}) in their propionylated forms. These findings corroborated the conclusions drawn from 2D-NMR analyses (HSQC and HMBC), validating the presence of native γ-acetylated lignin units.

The information obtained from DFRC (and DFRC') analysis, encompassing the proportions of lignin monomeric units, as well as tricin, alongside the percentages of γ-acetylated and γ-*p*-coumaroylated lignin units, are comprehensively presented in Table 4. The G-units were found to be the most predominant, comprising 59.5 % of the overall lignin units, followed by the S-units at 37.9 %, and with smaller quantities of H-units (2.6 %). The DFRC (and DFRC') data indicate that Ac and *p*CA are the main γ-acylating groups within tritordeum straw lignin, with the former being the predominant one. As occurs in other grasses, *p*-coumarates exhibited a preference for attaching to S-units, where 5.4 % of all S-units were found to be *p*-coumaroylated. In contrast, only minimal traces of G-units were observed to be *p*-coumaroylated, a

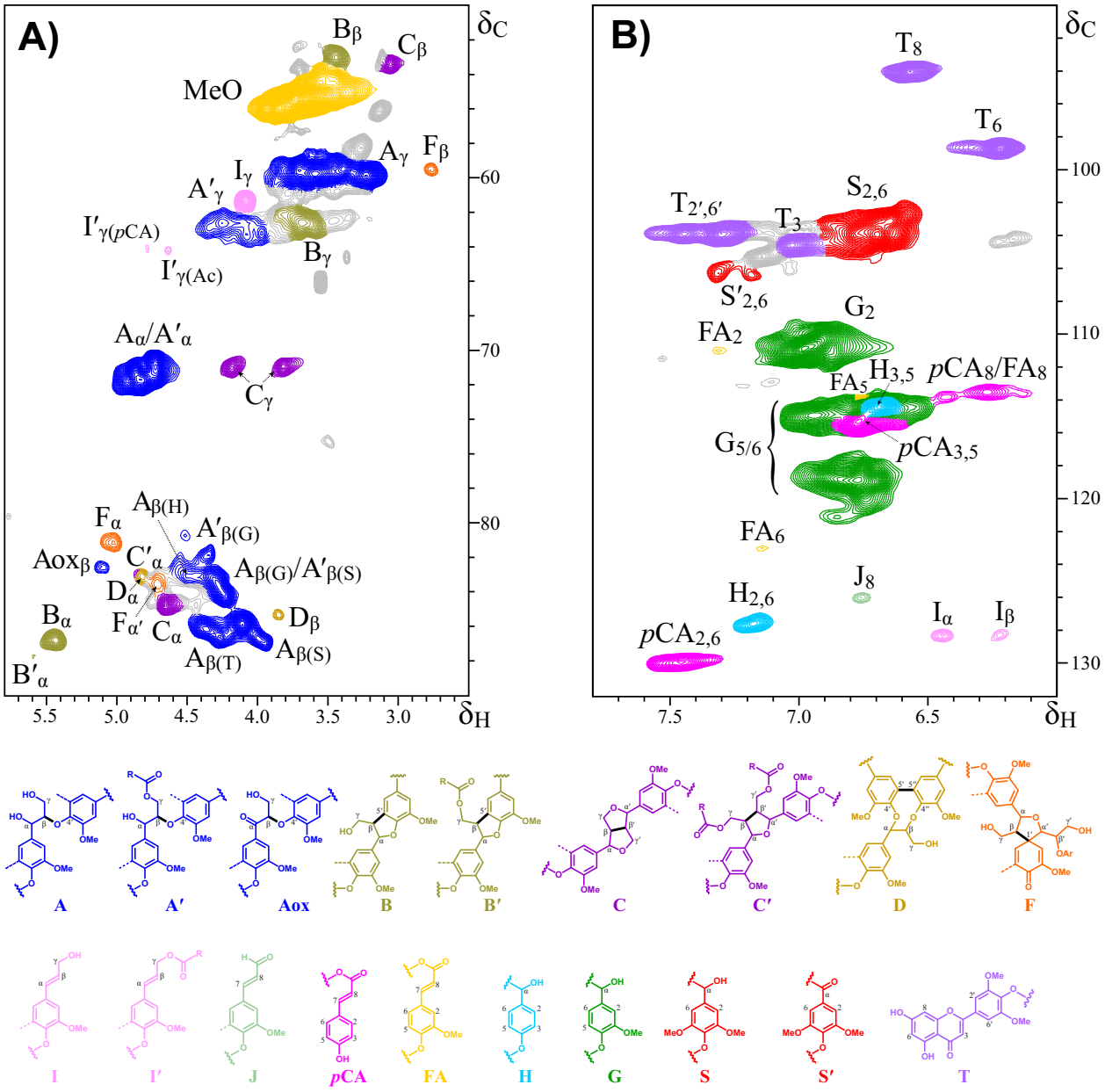

Fig. 2. Aliphatic-oxygenated (**A**) and aromatic/unsaturated (**B**) sections of the HSQC spectrum of the MWL isolated from tritordeum straw. At the bottom, lignin substructures identified, including: β–*O*–4′ alkyl-aryl ethers (A), γ-acylated β–*O*–4′ alkyl-aryl ethers (A′), Cα-oxidized β–*O*–4′ alkyl-aryl ethers (Aox), β–5′ phenylcoumarans (B), γ-acylated β–5′ phenylcoumarans (B′), β–β′ resinols (C), γ-acylated β–β′ tetrahydrofurans (C′), 5–5′ dibenzodioxocins (D), β–1′ spirodienones (F), cinnamyl alcohol end-groups (I), γ-acylated cinnamyl alcohol end-groups (I′), cinnamaldehyde end-groups (J), *p*-coumarates (*p*CA), ferulates (FA), *p*-hydroxyphenyl units (H), guaiacyl units (G), syringyl units (S), Cα-oxidized syringyl units (S′), and tricin (T), are represented.

phenomenon consistent with their parent lignins in wheat and barley [9,34]. However, unlike *p*-coumarates, acetates exhibited a preference for attachment to G-units, with 14.1 % of all G-units showing acetylation, while only 2.7 % of S-units were acetylated. This pattern is consistent with observations in lignins from other grass species [9,22,23,39,40], but it diverges from the typical acetylation preference found in most plants, where acetylation primarily targets S-lignin units [39,41,42]. This observation suggests that the enzymes responsible for monolignol acetylation in grasses exhibit a greater preference for coniferyl alcohol over sinapyl alcohol, as previously proposed [23,40]. Finally, the amounts of tricin released upon DFRC accounted for nearly 23.6 % of the total lignin units connected through β−ether linkages, aligning with the findings from HSQC analysis, which also indicated substantial tricin integration into the lignin structure.

3.3. Occurrence and quantification of lignin-incorporated tricin

As indicated above, 2D-HSQC NMR revealed a substantial presence of tricin within tritordeum straw lignin, accounting for around 24 % of the total lignin content. Likewise, DFRC also indicated that tricin accounted for nearly 23.6 % of the total lignin units participating in β−ether bonds. The HMBC spectrum provided unequivocal information on how tricin was integrated into the lignin structure. A clear correlation between the tricin $C_{4'}$ carbon at δ_C 139.5 with the H_β proton of β−*O*−4′ ether substructures (δ_H ~ 4.2–4.4) definitively demonstrated that tricin is connected to the lignin backbone via 4′–*O*–β ether linkages (Fig. 6). Interestingly, two distinct HMBC signals of the tricin $C_{4'}$ with protons at δ_H 4.26 and 4.35 ppm were clearly observed and perfectly matched the HSQC signals assigned to tricin involved in β−*O*−4′ structures with

Table 3
HSQC semiquantitative analysis of tritordeum straw MWL, including the lignin linkages, end-groups, γ-acylation degree, lignin units (H, G, S), as well as ferulate (FA), *p*-coumarates (*p*CA), and tricin (T) moieties.

Linkages (%)[a]	
β–*O*–4′ alkyl-aryl ethers (**A**/**A**′)	78
β–5′ phenylcoumarans (**B**/**B**′)	11
β–β′ resinols (**C**)	4
β–β′ tetrahydrofurans (**C**′)	1
5–5′ dibenzodioxocins (**D**)	2
β–1′ spirodienones (**F**)	4
End-groups (%)[a]	
Cinnamyl alcohol end-groups (**I**/**I**′)	7
Cinnamaldehyde end-groups (**J**)	2
Degree of γ-acylation (%)	14
Aromatic units	
H (%)	3
G (%)	61
S (%)	36
S/**G** ratio	0.6
Tricin (**T**, %)[b]	24
p-Hydroxycinnamates[b]	
p-coumarates (*p*CA, %)[b]	6
Ferulates (FA, %)[b]	1

[a] Estimated as molar percentage of the total linkages (**A**–**F**).
[b] T, FA and *p*CA contents as molar percentages of lignin content (**H** + **G** + **S** = 100).

monolignols [27,43]. This observation is particularly noteworthy as previous studies on lignin incorporating tricin had only reported one of these signals [9,44]. The presence of both signals in our study can be attributed to the *threo* and *erythro* isomers.

To gain further insights into the composition of the lignin of tritordeum straw, as well as to obtain an accurate estimation of the amount of tricin incorporated into this lignin, the MWL preparation was analyzed by ^{31}P NMR spectroscopy. This NMR technique is a well-established and effective technique renowned for its ability to discern and measure diverse hydroxyl groups within lignin, including carboxylic, aliphatic, and phenolic groups [30,45]. In addition, recent studies have also demonstrated the applicability of ^{31}P NMR for identifying and quantifying flavonoids, such as tricin, in different types of lignins [31,32]. The ^{31}P NMR spectrum of tritordeum straw lignin, accompanied by the assignment of the primary signals, is depicted in Fig. 7 and the quantitation of various hydroxyl groups, including those of tricin, is presented in Table 5. The dominant peaks observed in the ^{31}P NMR spectrum of tritordeum straw MWL were attributed to aliphatic hydroxyls, falling within the range of 145.0–150.0 ppm. These aliphatic hydroxyl groups of the lignin side-chain accounted for a total of 3.88 mmol/g. Additionally, phenolic hydroxyl groups were also clearly detected in the spectrum, appearing in the region of 137–145 ppm. The phenolic hydroxyls accounted for 1.73 mmol/g and encompassed hydroxyl groups derived from lignin units (H, G, G_5-condensed, and S), along with those originating from *p*CA and tricin. Among the phenolic hydroxyl groups, those from G-lignin units were the most abundant and

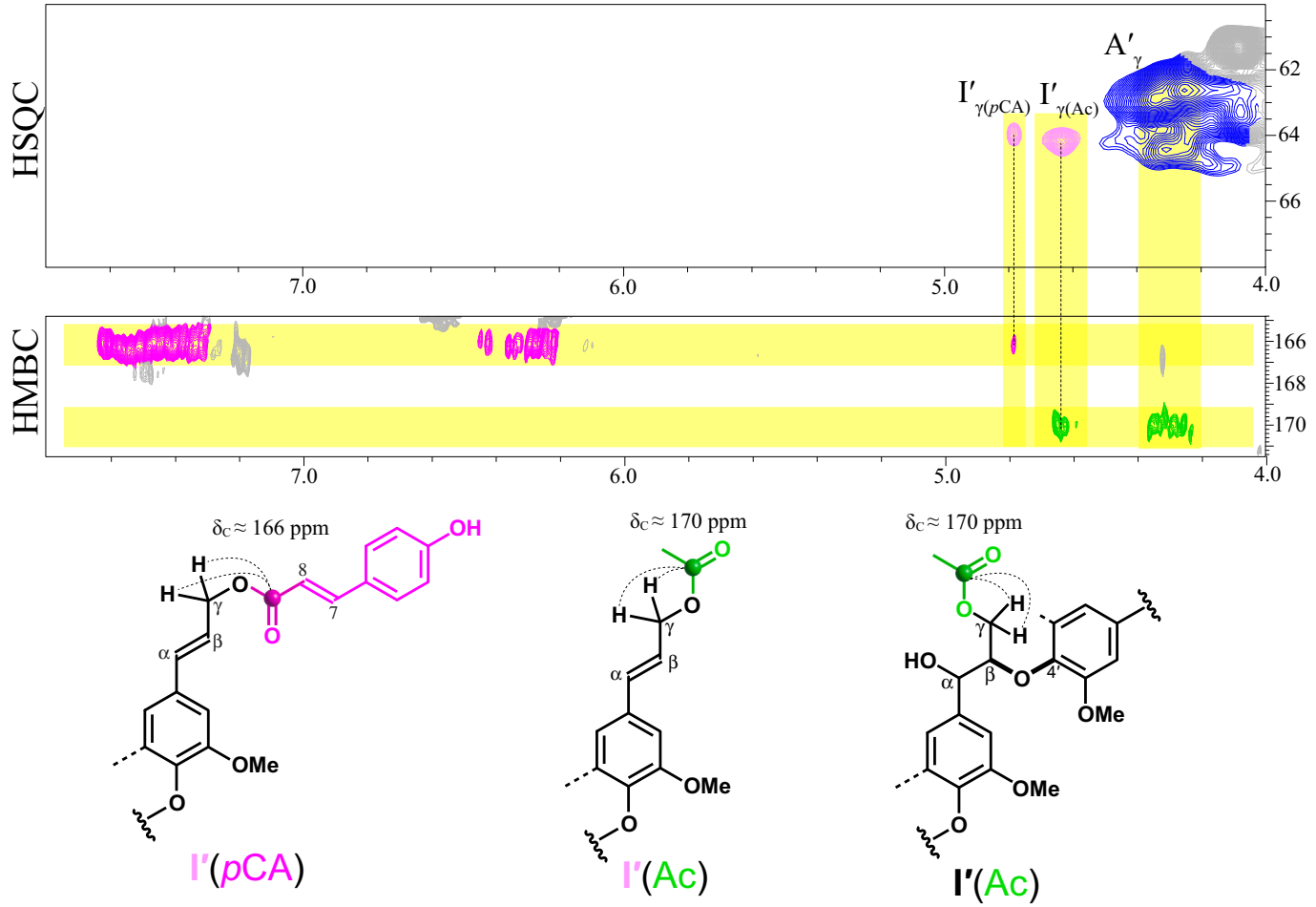

Fig. 3. Section of the HMBC spectrum (δ_C/δ_H 165–171/4.0–7.8) and appropriate section of the HSQC spectrum (δ_C/δ_H 60–68/4.0–7.8) of the MWL isolated from tritordeum straw showing the main correlations for the carbonyl carbons of γ-acylating groups (magenta for *p*-coumarates and green for acetates). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

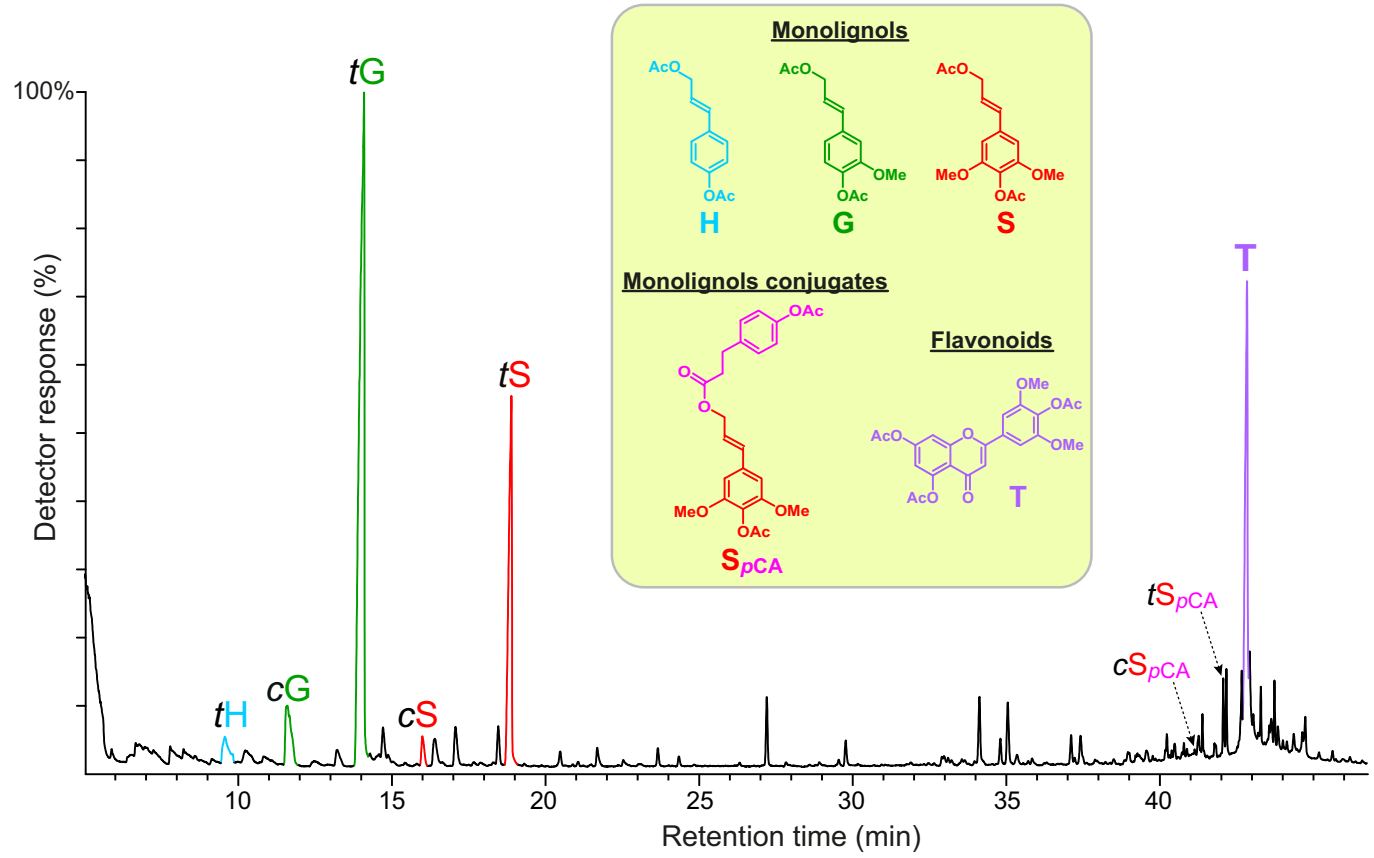

Fig. 4. Total-ion chromatogram of the DFRC degradation products released from the MWL isolated from tritordeum straw (as their acetate derivatives). The chromatogram highlights the presence of various compounds, including monolignols (H, G, and S), monolignol-*p*-coumarates (*cis*- and *trans*-sinapyl-dihydro-*p*-coumarates, cS_{pCA}, and tS_{pCA}), and tricin (T).

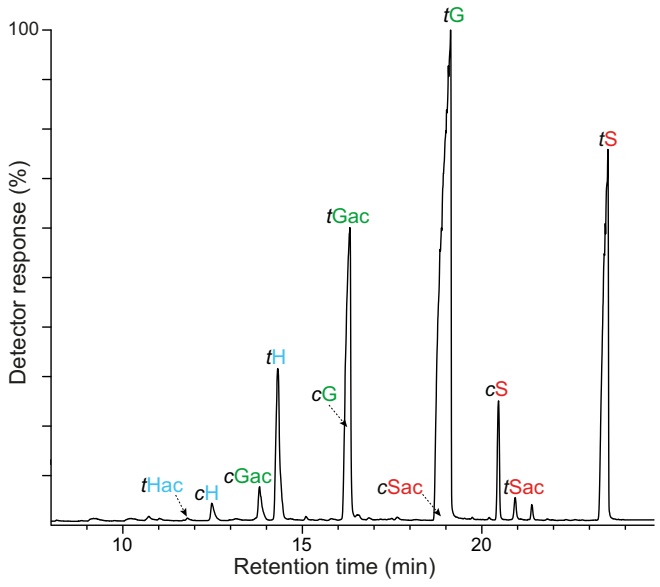

Fig. 5. Reconstructed ion chromatogram (sum of the ions at *m/z* 192 + 206 + 222 + 236 + 252 + 266, which are characteristic of the γ-acetylated and γ-OH H, G, and S lignin units) of the DFRC′ degradation products of the MWL isolated from tritordeum straw. *c*H, *t*H, *c*G, *t*G, *c*S, and *t*S are the normal (γ-OH) *cis*- and *trans*-*p*-hydroxyphenyl, guaiacyl, and syringyl monomers, respectively (as their propionylated derivatives). *t*Hac, *c*Gac, *t*Gac, *c*Sac, and *t*Sac are the originally γ-acetylated *cis*- and *trans*-*p*-hydroxyphenyl, guaiacyl, and syringyl monomers, respectively (as their propionylated derivatives).

accounted for 0.81 mmol/g, whereas the phenolic hydroxyl groups of S-lignin units were barely detected and only accounted for 0.12 mmol/g. In the case of phenolic groups of H-lignin units and *p*CA, whose signals overlap between them, as well as with that from tricin 5-OH [30], it was not possible to estimate their amounts individually. Carboxylic-OH groups, appearing at 134.5–135.5 ppm, were also detected, although their intensities were comparatively lower (0.10 mmol/g). Unlike the tricin 5-OH peak that appears overlapped with other hydroxyphenyl hydroxyls peaks, the tricin 7-OH peak appeared well-resolved and separated (Fig. 7) and therefore it was used for determining the tricin content (0.55 mmol/g). By considering a ratio of one mole of tricin 7-OH per mole of tricin and taking into account the total lignin content in tritordeum straw (15.5 %, dry weight), we were able to estimate that the tricin integrated into the tritordeum straw lignin was approximately 30 mg/g of straw (on a dry weight-basis). This estimation significantly surpasses the tricin content observed in the wheat and barley, which are the parental plants of tritordeum [18].

A remarkable observation was the absence of signals within the 142.0–143.0 ppm range, corresponding to the 4′-OH of free tricin [31], which confirms that tricin is not present in free from. Instead, all the tricin present in the MWL preparation is linked to the lignin polymer via 4′–*O*–β ether bonds, consistent with the results obtained from the 2D-HMBC analysis. Considering that tricin exclusively binds to the lignin polymers through tricin-4′–*O*–β-ethers, various chemical lignin degradation methods capable of cleaving β-ethers (such as acidolysis, thioacidolysis, and DFRC) result in the release of tricin from the lignin matrix [11]. Other potentially effective methods for releasing tricin from the lignin polymer could be autohydrolysis [46] and steam explosion [47], which are known for cleaving ether linkages. Conversely, treatments in alkaline aqueous solution (e.g. kraft method) may not be

Table 4
Relative molar abundance of H-, G-, and S-lignin units with non-acylated and acylated γ-OH (with acetates and *p*-coumarates) and tricin (T), estimated from DFRC (and DFRC′) degradation of the MWL isolated from tritordeum straw. The percentages of acetylated and *p*-coumaroylated lignin units are also shown.

	H	G	G_{ac}	G_{pCA}	S	S_{ac}	S_{pCA}	T[a]	$\%G_{ac}$[b]	$\%G_{pCA}$[c]	$\%S_{ac}$[d]	$\%S_{pCA}$[e]
Tritordeum MWL	2.6	51.1	8.4	tr	34.9	1.0	2.0	23.6	14.1	tr	2.7	5.4

[a] T molar content referred as to the percentage of total lignin units ($H + G + G_{ac} + G_{pCA} + S + S_{ac} + S_{pCA} = 100$).
[b] $\%G_{ac}$ is the percentage of acetylated G units (G_{ac}) with respect to the total G units ($G + G_{ac} + G_{pCA}$).
[c] $\%G_{pCA}$ is the percentage of *p*-coumaroylated G units (G_{pCA}) with respect to the total G units ($G + G_{ac} + G_{pCA}$).
[d] $\%S_{ac}$ is the percentage of acetylated S units (S_{ac}) with respect to the total S units ($S + S_{ac} + S_{pCA}$).
[e] $\%S_{pCA}$ is the percentage of *p*-coumaroylated S units (S_{pCA}) with respect to the total S units ($S + S_{ac} + S_{pCA}$).

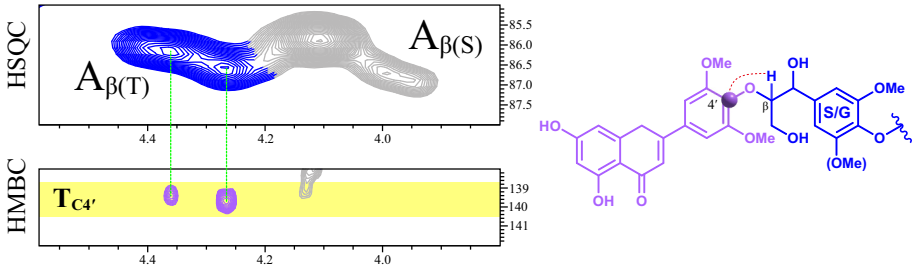

Fig. 6. Section of the HMBC spectrum (δ_C/δ_H 138–142/3.8–4.6) and appropriate section of the HSQC spectrum (δ_C/δ_H 85–88/3.8–4.6) of the MWL isolated from tritordeum straw showing the correlations between the $C_{4'}$ of tricin with the H_β of the monolignol side-chains involved in β−*O*−4′ structures.

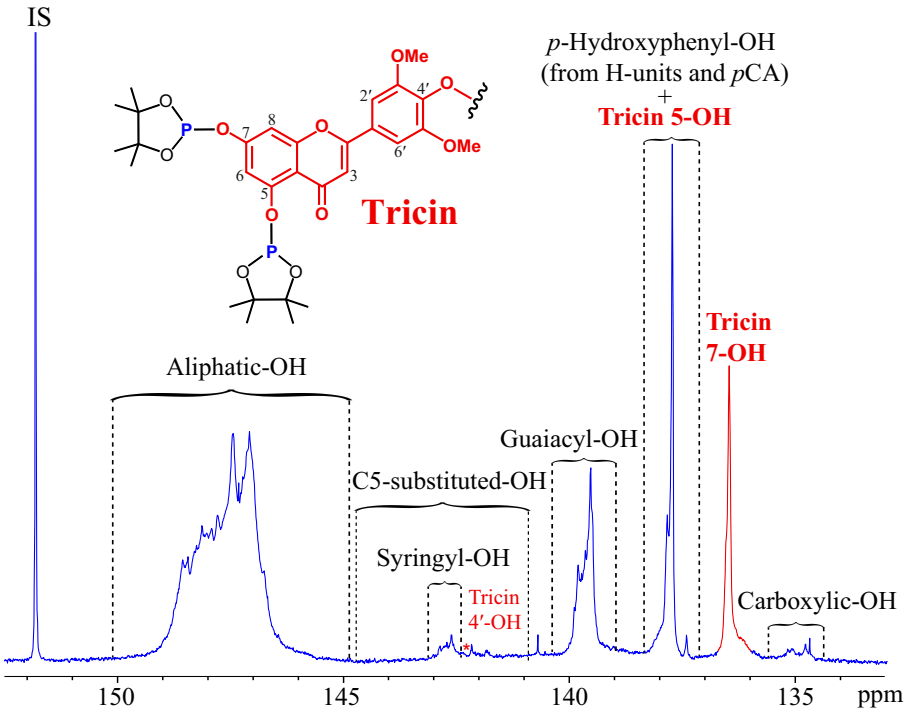

Fig. 7. ^{31}P NMR spectrum of tritordeum straw MWL phosphitylated with TMDP and using NHND as an internal standard (IS) for quantification.

suitable, as flavonoids can undergo degradation (autoxidation) under these conditions [48].

Furthermore, the examination of tritordeum straw MWL using gel permeation chromatography (GPC) presented compelling evidence that tricin is an inherent constituent of the lignin polymer, as no peaks corresponding to low molecular weight compounds (<500 g/mol) were observed in the GPC chromatogram (**Fig. S1**). Hence, the GPC data conclusively ruled out the possibility that tricin might be present either in its free form or as a low molecular weight flavolignan within the MWL preparation. Instead, it firmly established that tricin is integrated into the lignin polymer, as previously indicated by the ^{31}P NMR analysis. The GPC analysis of tritordeum straw MWL revealed a weight-average (M_w) molecular weight of 3660 g/mol and a number-average (M_n) molecular weight of 2480 g/mol, accompanied by a remarkably low polydispersity index ($M_w/M_n = 1.5$), indicating an exceptional degree of uniformity within this lignin. An intriguing observation arises from the fact that each lignin chain can only accommodate one molecule of tricin. Consequently, a higher tricin content corresponds to a lower molecular weight of the lignin. This observation offers an explanation for the relatively low M_w of tritordeum lignin when compared to other lignins

Table 5
Aliphatic, phenolic (Syringyl + Guaiacyl + *p*-Hydroxyphenyl + C_5-substituted-OH + tricin), and carboxylic hydroxyl group contents (mmol OH/g lignin) in the MWL isolated from tritordeum straw as determined by quantitative ^{31}P NMR.

	Content (mmol OH/g MWL)
Aliphatic-OH	3.88
Phenolic-OH	1.73
G-OH	0.81
S-OH	0.12
p-Ar-OH (H-OH + *p*CA-OH)	0.17
C5-substituted-OH	0.08
Tricin-OH	0.55
Carboxylic-OH	0.10

with low or no tricin content [49]. Furthermore, this phenomenon extends to other tricin-enriched lignin sources, exemplified by lignin derived from wheat straw and vanilla aerial roots, among others, where their M_w values align with those of tritordeum lignin [32,47].

4. Conclusions

The lignin extracted from tritordeum straw underwent a comprehensive analysis, employing cutting-edge analytical techniques such as Py-GC/MS, 2D-NMR, DFRC, and GPC. The analysis revealed that this lignin was predominantly comprised of G- and S-lignin units, mainly interconnected by β–*O*–4′ linkages. Additionally, tritordeum straw lignin exhibited partial γ-acylation with Ac and *p*CA groups, with the former mainly acylating the γ-OH of S-units, while the latter preferentially acylates G-units. A remarkable discovery was the high abundance of tricin integrated into this lignin, accounting for up to 24 % of the total lignin content, as revealed by 2D-HSQC-NMR. Quantitatively, and considering that the MWL preparation is representative of the total native lignin in the straw, up to 30 g of lignin-incorporated tricin per kilogram of tritordeum straw were found, as estimated from ^{31}P NMR data. In the NMR analysis, it was elucidated that tricin exclusively formed 4′–*O*–β ether linkages with the lignin backbone, placing it as a pendant group at the starting point of the lignin chains. The terminal positions of tricin linked via β-ether bonds, along with its high value and numerous potential industrial applications, make tritordeum straw a promising raw material for obtaining this valuable compound. Further investigations should explore diverse methodologies aimed at releasing tricin from the lignin polymer.

Funding

This research was made possible through financial support from MCIN/AEI/10.13039/501100011033 (project PID2020-118968RB-I00) and EU-NextGenerationEU/PRTR (project CPP2021-008449). Additionally, this study was made possible through the CSIC project PIE-202040E185. Furthermore, the contributions of JB and MJR were financially supported by FPI grants PRE2021-099559 and PRE2018-083267, respectively.

CRediT authorship contribution statement

Javier Benito: Data curation, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Gisela Marques:** Investigation. **Mario J. Rosado:** Investigation. **Francisco Barro:** Funding acquisition, Resources. **Ana Gutiérrez:** Funding acquisition, Methodology, Project administration, Resources. **José C. del Río:** Resources, Writing – review & editing. **Jorge Rencoret:** Conceptualization, Data curation, Funding acquisition, Project administration, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

The authors extend their gratitude for the invaluable technical assistance provided by Dr. Manuel Angulo at SGI-CITIUS for acquiring the NMR spectra presented in this study. Additionally, the authors wish to express their appreciation to Cristóbal Martínez and Alba Martínez from IFAPA-Córdoba and Vivagran SL, respectively, for their dedicated efforts in cultivating and harvesting the specific tritordeum plants meticulously chosen for this research.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2024.129694.

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