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^{31}P NMR Characterization of Tricin and Its Structurally Similar Flavonoids

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Abstract: Tricin, a flavonoid metabolite, has been recently identified as a component of lignin in select monocot plants. This finding has initiated consideration on updating the lignin biosynthesis pathway. We here report a rapid method of determination of tricin in corn stover lignin, based on ^{31}P nuclear magnetic resonance (NMR) spectroscopy by phosphitylating with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (TMDP). Nine other flavonoids, with similar structure to tricin, have also been examined using the current method. The application of ^{31}P NMR enables rapid identification of tricin-like flavonoids in the heterogeneous lignin polymer. The well resolved spectroscopic peaks from these derivatized flavonoids and lignin functional groups provide important information for the determination of flavonoids individually or their association with lignin.

Lignin, a heterogeneous biopolymer with aromatic structure, is found in most terrestrial plants with abundant occurrence. Due to its sustainability, high carbon content and high aromaticity, lignin attracts tremendous attention as a promising resource for the production of fuels, chemicals, and bio-derived materials.[1] However, the valorization of lignin and conversion of lignocellulosic biomass to biofuels and valuable chemicals are restricted largely by the chemical recalcitrance and structural complexity of lignin.[2]

Nuclear magnetic resonance spectroscopy (NMR)-based tools with sufficient resolution and dispersion have enabled the analysis of lignin structural details. It is now well established that ^{13}C NMR and two-dimensional (2D) HSQC techniques can readily determine the basic components of lignin (e.g., syringyl, guaiacyl, *p*-hydroxyphenyl), oxidized functionality, and interunit linkages.[3] It is also routinely used to detect non-lignin components, such as carbohydrates that occasionally accompany isolated lignin samples.[4] NMR methodologies have also been crucial for the identification of non-conventional lignin structures, such as dihydroconiferyl alcohol and C-lignin (catechyl lignin) in seeds of certain plant species.[5] The recent observation of tricin, a flavonoid metabolite with a 15-carbon skeleton, including phenyl rings (A and B) and one heterocyclic ring (C) (Figure 1), has been revealed by 2D NMR spectroscopic analysis.[6] The coupling of tricin with monolignols in biomimetic radical conditions suggests the feasibility of tricin's involvement in lignification in at least a few monocots.[7] Although this metabolite has been extensively studied due to its biological and pharmaceutical functions, the observation of covalent linkage of tricin to lignin in some monocot species leads to the question of how lignification and flavonoid biosynthesis merge in plants.[8] Therefore, a rapid and accurate determination of the flavonoid subunit can provide useful information on lignin structure that is ultimately required to update the lignification pathway. In addition, the proposed initiation role of tricin in grass lignin biosynthesis provides a potential strategy to manipulate lignification for bioenergy crop improvement.[9]

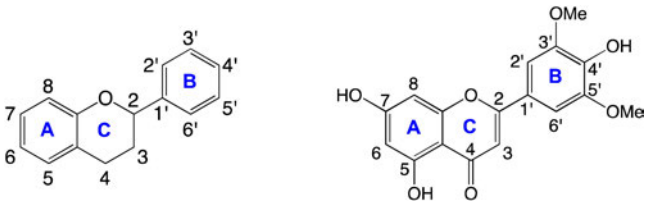

Figure 1. Structure of basic flavonoid (left) and flavone tricin (right).[8,10]

A complementary approach in which lignin hydroxyl groups (OHs) are phosphitylated with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (TMDP) and determined using ^{31}P NMR after derivatization (Scheme 1) has been widely used on qualitative and quantitative analysis of different types of lignin OHs.[11] With suitable phosphitylation, different OHs deriving from aliphatic, various phenolic, and carboxylic parts of lignin, have been well resolved and identified using ^{31}P NMR spectroscopy.[11b,12] Compared with ^{13}C and 2D HSQC NMR, ^{31}P NMR has a few unique advantages in lignin structural analysis, such as small amount of sample needed, relatively short experimental

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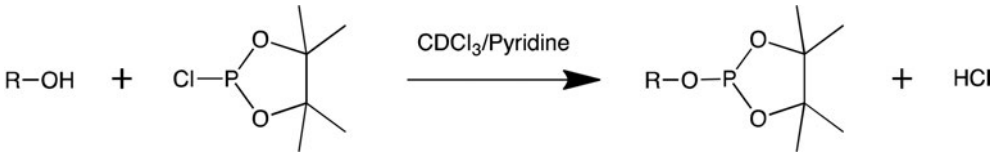


Scheme 1. Phosphitylation of hydroxyl group in flavonoids/lignin (R) with TMDP.

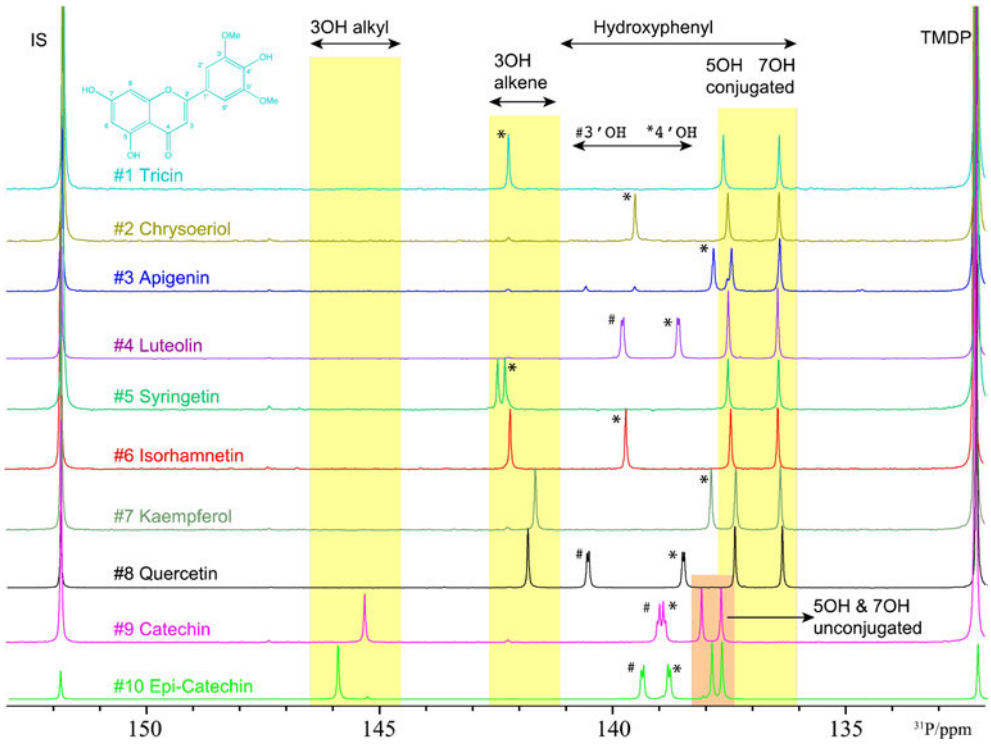


Figure 2. ^{31}P NMR spectra of tricin and tricin-like flavonoids phosphitylated with TMDP. IS: internal standard.

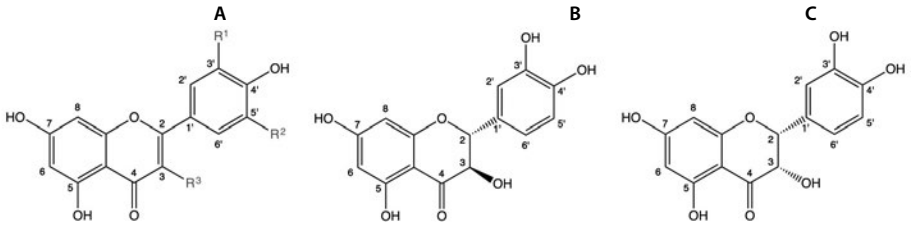
time, high sensitivity, and high resolution of signals.[11b,13] It has been successfully employed to detect and quantify phenolic compounds, including two flavonoids—apigenin and luteolin, in the polar fraction of virgin olive oil.[14] More recently, ^{31}P NMR has been used to identify tricin in wheat straw lignins.[15] This rapid methodology provides wide application and important information in characterizing lignin compositional units, which is vital in the era of developing bio-based economies.

In this work, we attempt initially to examine tricin and 9 other structurally similar tricin-like flavonoids and provide a small database for diagnosing OHs derived from flavonoids. We also develop a qualitative method that can detect the presence of tricin in lignin using ^{31}P NMR. With diagnostic peaks, the OHs from tricin (and tricin-like flavonoids) units have been identified and distinguished from other phenolic OHs in lignin samples isolated from poplar and corn stover.

We phosphitylated tricin and 9 other tricin-like flavonoids and analysed them using ^{31}P NMR spectroscopy. The OHs derived from alkyl, alkene, and hydroxyphenyl were well resolved in the ^{31}P NMR spectra (Figure 2) and their chemical shifts are listed in Table 1. The chemical shift of phosphitylated alkyl and alkene OH at the 3 position of ring-C were distinguished with sharp peaks located at the narrow range of δ 145 to 146 ppm (compounds 9 and 10) and δ 141.5-142.5 ppm (compounds 5, 6, 7, and 8), respectively. In contrast, the hydroxyphenyl OHs had a relatively large variation of chemical shift (from δ 136.3 to 142.2 ppm) and the peak locations were significantly affected by its chemical environment. For instance, the derivatized OH on the positions 5 and 7 of ring-A which conjugated with the double bonds in ring-C (compounds 1 to 8) centred around δ 136.4 (7OH) and 137.4 (5OH), whereas the same OHs in ring-A without conjugation (compounds 9 and 10) had about a 1 ppm downfield shift (red region in Figure 2). The phosphitylated OHs at the 5 and 7 positions were differentiated according to their proximity to the deshielding carbonyl group at the position 4. With less bonded distance, the phosphitylated 5OH is expected to have a downfield shift.[16]

In addition, the phosphitylated OH on ring-B was remarkably sensitive to the substituents around the labile hydrogen center. For instance, tricin (1), chrysoeriol (2), and apigenin (3) have identical structure except for the difference in the number of methoxyl groups (OMe) substituted in the *ortho* to the phenolic OH at the 4′ position (marked with * in Figure 2). Substituent of OMe resulted in significant downfield shifts of 1.7 ppm (2 vs 3 or 7) for one OMe substitution and another 2.7 ppm (1 vs 2 or 6) for the 2nd OMe substitution. This observation of sensitive downfield shift towards *ortho* substitution is consistent with the previous study.[11a] When the *ortho* position was substituted with another OH at the 3′ position,

Table 1. ^{31}P NMR chemical shifts of phosphitylated flavonoids. (N/A: not applicable)



#	Compounds	Structure	OH_3	OH_5	OH_7	$OH_{4'}$	$OH_{3'}$
1	Tricin	A: $R^1 = R^2 =$ OMe; $R^3 =$ H	N/A	137.6	136.4	142.2	N/A
2	Chrysoeriol	A: $R^1 =$ OMe; $R^2 = R^3 =$ H	N/A	137.5	136.4	139.5	N/A
3	Apigenin	A: $R^1 = R^2 = R^3 =$ H	N/A	137.4	136.4	137.8	N/A
4	Luteolin	A: $R^1 =$ OH; $R^2 = R^3 =$ H	N/A	137.5	136.5	138.6	139.8
5	Syringetin	A: $R^1 = R^2 =$ OMe; $R^3 =$ OH	142.5	137.5	136.4	142.3	N/A
6	Isorhamnetin	A: $R^1 =$ OMe; $R^2 =$ H; $R^3 =$ OH	142.2	137.4	136.4	139.7	N/A
7	Kaempferol	A: $R^1 = R^2 =$ H; $R^3 =$ OH	141.6	137.3	136.4	137.9	N/A
8	Quercetin	A: $R^1 =$ OH; $R^2 =$ H; $R^3 =$ OH	141.8	137.4	136.4	138.5	140.5
9	Catechin	B	145.3	138.1	137.7	138.9	139.0
10	Epi-catechin	C	145.9	137.9	137.7	138.8	139.4

the peak of derivatized 4'OH had shown in doublet peaks (4, 8, 9, and 10). Both the doublet signals deriving from the four compounds reflected the unusual homoallylic coupling ($^5J_{pp}$ = 6.0-8.2 Hz), which are consistent with previous observation in ^{31}P NMR spectra of quercetin.[16] The chemical shift of 3'OH and 4'OH derivatives span into the region from δ 138.5 to 140.5 ppm. The signals corresponding to 3'OH (marked with #) and 4'OH (marked with *) positions in compounds 4 and 8 were differentiated by comparing their spectra with similar compounds 3 and 7 (Figure 2). The signals of downfield chemical shift at 139.8 ppm in compound 4 and 140.5 ppm in compound 8, which were assigned to 3'OH, were missing in compounds 3 and 7. These tentative assignments are consistent with quercetin in previous literature.[16] The relative chemical shifts of 3'OH and 4'OH were, therefore, used to identify their assignments in compounds 9 and 10. ^{31}P NMR also revealed stereochemical details of flavonoids. For instance, catechin (9) and epi-catechin (10) in *trans*- and *cis*-configuration, respectively, could be well identified due to their characteristic peaks on 3OH, 3'OH, and 4'OH.

Previous studies have shown that the incorporation of tricin units in lignin was typically reported in monocots, such as wheat, maize, and bamboo, rather than dicots or gymnosperms.[8] In order to identify the diagnostic peaks deriving from the tricin in lignin, two cellulolytic enzyme lignins (CELs) that were reported with and without tricin incorporation were respectively isolated and used for comparison: poplar (*Populus trichocarpa*) that showed no tricin incorporation into lignin (lignin-POP), and corn (*Zea mays*) stover with tricin incorporated lignin (lignin-CS). The colored contours in Figure 3 depict the difference of compositional units in the lignins between poplar and corn stover, with the light yellow blocks highlighting the tricin units. According to the results revealed by 2D NMR spectroscopic analysis,[7,17] the peaks with δ 94.1/6.57, δ 98.8/6.23, δ 104.0/7.30, and δ 104.5/7.04 ppm in the light yellow blocks regions of the HSQC spectrum of corn stover lignin were assigned to the 8, 6, 2'/6', and 3 positions of tricin, respectively. By contrast, no tricin signals were detected in the poplar lignin by 2D HSQC NMR.

We next investigated the diagnostic peak of tricin in lignin using ^{31}P NMR. After phosphitylation with TMDP, the ^{31}P spectra of both poplar and corn stover lignin are primarily consisted of derivatives from aliphatic, C_5 substituted, guaiacyl, hydroxyphenyl, and carboxylic OH (lignin-POP and lignin-CS in Figure 4). The distinguished peaks in corn stover lignin (lignin-CS in blue) located at δ 136.4 and 137.8 ppm corresponded to hydroxyphenyl hydroxyls, with no peak detected at δ 136.4 ppm and a strikingly reduced peak abundance around δ 137.8 ppm in poplar lignin (lignin-POP in red). By comparing the chemical shifts of these two peaks with phosphitylated tricin (Figure 4), they likely corresponded to the OHs at 7 and 5 positions in ring-A of tricin unit, or at least tricin-like flavone type unit. The 4'OH of tricin was not detected in the lignin samples as the tricin unit was mainly connected to other lignin components through the ether bond at the 4' position.[7] Therefore, the ^{31}P NMR of TMDP derivatized lignin can be used to detect tricin or tricin-like units in lignin.

To further confirm this method in identifying tricin, we then mixed tricin and lignin-POP and lignin-CS, respectively, and derivatized the mixture for ^{31}P analysis (tricin mixed lignin-POP and tricin mixed lignin-CS in Figure 4). The spectra of tricin-lignin mixture after phosphitylation showed three diagnostic peaks at δ 136.4, 137.6, and 142.2 ppm, corresponding to free tricin. Consistent with previous spectra of tricin and lignin analysed independently, however, the tricin unit in corn stover lignin had 0.2 ppm downfield shift compared with the free tricin at the position 5. This difference was likely attributed to the substitution effects of bulky lignin on tricin. In addition, by comparing the spectrum of poplar lignin with that of corn stover lignin, we noted that poplar lignin with no tricin unit incorporated had no detectable phosphitylated 7OH at δ 136.4 ppm. However, poplar lignin showed a relatively strong peak at δ 137.8 ppm derived from other hydroxyphenyl hydroxyl (e.g., *p*-hydroxybenzoate) in lignin, which overlapped with the chemical shift of the 5OH of tricin unit. In the corn stover lignin, the peak appeared at δ 137.8 ppm was contributed probably from both the tricin unit and other hydroxyphenyl OH units (e.g., *p*-hydroxycoumarates). Therefore, it should be noted that the quantification of the OH for C_5 substituted, *p*-hydroxyphenyl, and carboxylic OH need to be careful for tricin contained lignin because the three tricin OH peaks are located in the range of these three groups.[11b,18]

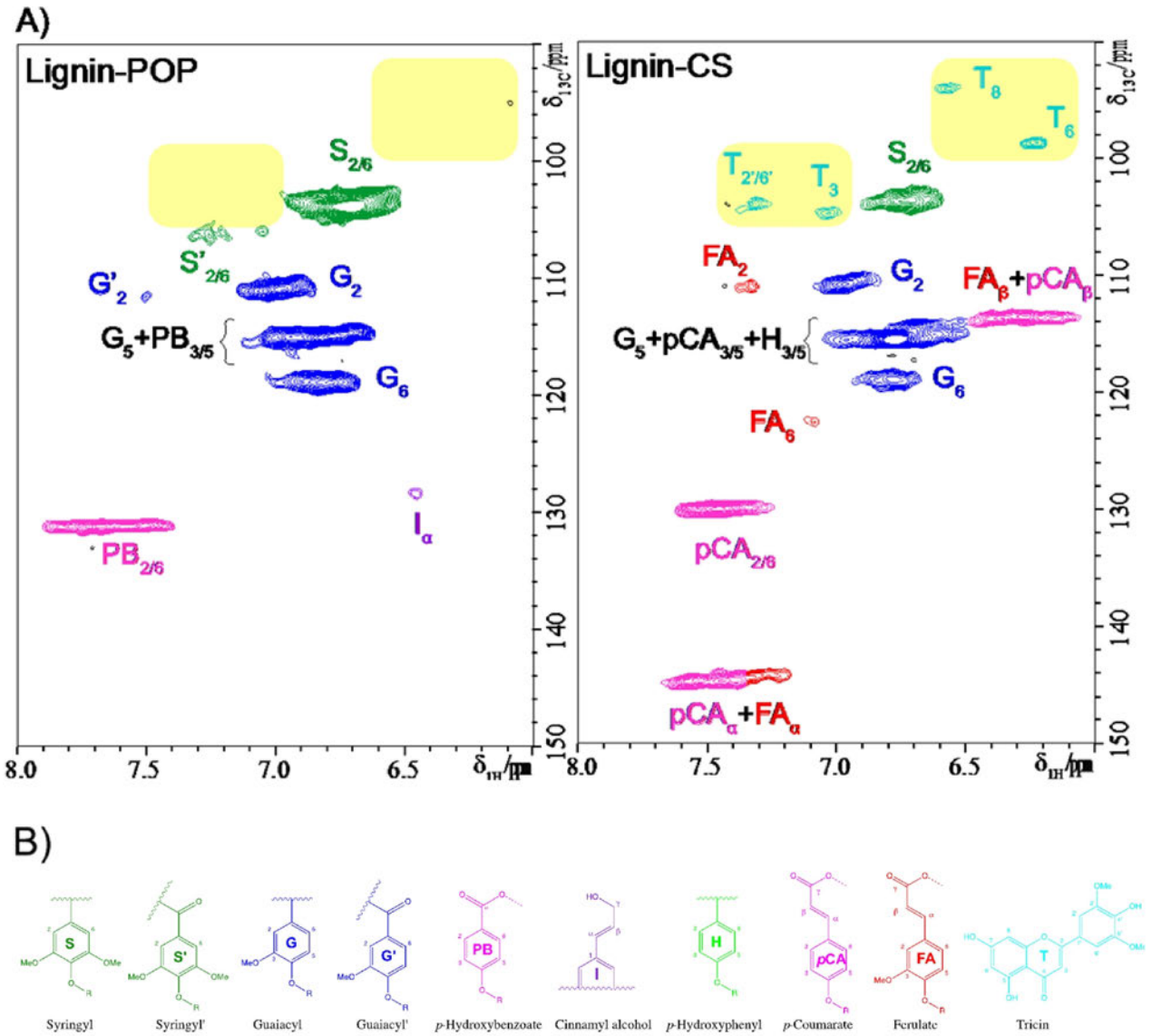


Figure 3. 2D NMR spectra of cellulolytic enzyme lignin (CEL) isolated from poplar (left) and corn stover (right). (A) Partial short-range ^{13}C-^{1}H (HSQC) correlation spectra (aromatic regions only) revealing (B) lignin unit compositions coded with color correspondingly.

In summary, we used ^{31}P NMR spectroscopy for identifying tricin and tricin-like flavonoids in lignin after derivatization with TMDP. By comparing the tricin-incorporated lignin from *Zea mays* and non-tricin-incorporated lignin from *Populus trichocarpa*, ^{31}P NMR spectroscopic analysis rapidly provided diagnostic peaks of tricin-like structure in lignin. The chemical shifts of tricin and other 9 tricin-like flavonoid derivatives in ^{31}P NMR were also recorded. While caution should be taken for the quantification of different types of OH for tricin-contained lignin due to some peaks overlapping, the good resolution and high sensitivity to the chemical environment of phosphitylated OHs can be exploited to detect and quantify flavonoids.

Supporting Information Summary

Experimental details and methods information could be found in the supporting materials.

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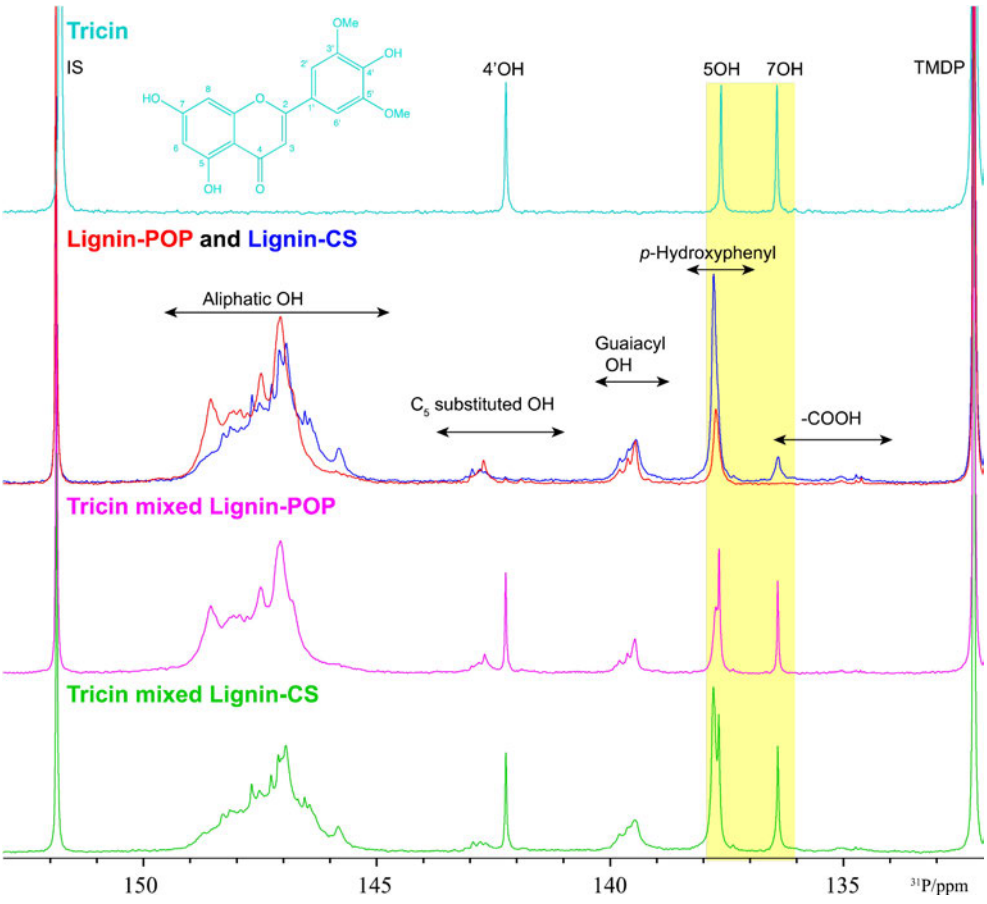

Figure 4. ^{31}P NMR spectra of tricin, lignin from poplar (lignin-POP) and corn stover (lignin-CS), mixture of tricin and poplar lignin and corn stover lignin. The range of lignin functional groups identification was marked according to literature. [11b,18] IS: internal standard; -COOH: carboxylic acid group.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: biomass · flavonoids · lignin · NMR spectroscopy · tricin

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