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Tannin Structural Elucidation and Quantitative ^{31}P NMR Analysis. 1. Model Compounds

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ABSTRACT: Tannins and flavonoids are secondary metabolites of plants that display a wide array of biological activities. This peculiarity is related to the inhibition of extracellular enzymes that occurs through the complexation of peptides by tannins. Not only the nature of these interactions, but more fundamentally also the structure of these heterogeneous polyphenolic molecules are not completely clear. This first paper describes the development of a new analytical method for the structural characterization of tannins on the basis of tannin model compounds employing an in situ labeling of all labile H groups (aliphatic OH, phenolic OH, and carboxylic acids) with a phosphorus reagent. The ^{31}P NMR analysis of ^{31}P-labeled samples allowed the unprecedented quantitative and qualitative structural characterization of hydrolyzable tannins, proanthocyanidins, and catechin tannin model compounds, forming the foundations for the quantitative structural elucidation of a variety of actual tannin samples described in part 2 of this series.

KEYWORDS: *tannins, ^{31}P NMR, proanthocyanidins, polyphenols, flavonoids*

INTRODUCTION

Among the secondary metabolites of plants, phenolic compounds range from the familiar flower pigments (anthocyanidins) to the complex phenolics of their cell walls (lignin).[1] The group of phenolic compounds known as tannins, however, is clearly distinguished from other plant secondary phenolics in their chemical and biological activities. After lignins, they are the second most abundant group of plant phenolics. Tannins are water-soluble polyphenolic compounds with molecular weights ranging from 500 to 20000 Da. The presence of a large number of phenolic hydroxyl groups enables them to form large complexes, mainly with proteins, alkaloids, and polysaccharides,[4,5] The ability to precipitate proteins distinghishes the tannins from most other natural phenolic compounds and forms the basis for their past and present use in the tanning industry.[2,3]

Tannins are widespread in the plant kingdom (pteridophytes, gymnosperms, and angiosperms) and are found in leaves, fruits, bark, and wood.[6] Tannins are stored in leaf, bud, seed, root, or stem tissues and are physically located in the vacuoles or surface wax.[7] They are responsible for the typical taste of astringency of fruits and leaves, making them less appetizing for herbivores. These compounds have a range of effects on various organisms, ranging from growth inhibition to toxicity.[8]

Tannins inhibit the growth of a number of microorganisms, resist microbial attack, and are recalcitrant to biodegradation.[9] Condensed tannins are more resistant to microbial attack than hydrolyzable tannins and are toxic to a variety of microorganisms.

Tannins do not constitute a unified chemical group, but display a variety of molecular structures. On the basis of their structures and properties, they are distributed into two major groups: hydrolyzable, which condensed tannins. Hydrolyzable tannins are gallic acid derivatives, which are classified as gallo- and ellagitannins.[10,11]

The major commercial hydrolyzable tannins are extracted from Chinese gall (*Rhus semialata*), sumac (*Rhus coriara*), Turkish gall (*Quercus infectoria*), tara (*Caesalpina spinosa*), myrobalan nuts (*Terminalia chebula*), and chestnut (*Castanea sativa*) (Table 1). Gallotannins are gallic acid esters of a core polyol, usually glucose. The simplest hydrolyzable prototannin is pentagalloyl glucose (*β*-1,2,3,4,6-pentagalloyl-*O*-D-glucopyranose) (PGG) (Figure 1A). PGG occurs in different isomers in which one aliphatic hydroxyl group is free and the other is present as a digalloyl ester (Figure 1B). Polygalloyl ester chains are formed by a meta- or para-depside bond involving a phenolic hydroxyl rather than an aliphatic hydroxyl group (Figure 1C). Simple gallotannins with up to 12 galloyl groups are present in tannins extracted from sumac or oak galls. Tannic acid is described as glucose pentagalloyl gallate, but it is actually a mixture of different isomers and partially galloylated glucose. Hydrolysis with strong acids converts gallotannins to gallic acid and the core polyol.

Ellagitannins are esters of hexahydrodiphenic acid (HHDP) (Figure 2) that in turn is generated by oxidative cross-linking of two galloyl groups. Upon hydrolysis the ellagitannins release HHDP, which in turn lactonizes to ellagic acid. Coupling occurs preferentially between C-4/C-6 (eugeniin) and C-2/C-3 (casuarictin) (Figure 2). Other isomers such as castalgin and vescalgin (Figure 2C) are found only in a few plants. In oak and chestnut ellagitannins the pyranose ring is opened and the galloyl groups are further cross-linked.[12]

Condensed tannins, better known as proanthocyanidins, are polymeric flavonoids. They are usually more abundant in tree barks and woods than their hydrolyzable counterparts. The important commercial condensed tannins are extracted from wattle (*Acacia mollissima* and *Acacia mearnsii*), quebracho

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Table 1. Tannin Occurrence

	hydrolyzable tannins		catechin tannins	condensed tannins
chemical structure	gallotannins, e.g., tannic acid; yield gallic acid and glucose upon hydrolysis	ellagitannins; yield ellagic acid and glucose upon; hydrolysis	catechin and epicatechin gallates; yield catechin/epicatechin and gallic acid on hydrolysis; properties of hydrolyzable and condensed tannins	polymeric proanthocyanidins; yield monomeric flavonoids such as flavan-3,4-diols and flavan-3-ols on oxidation
sources	tara pods (*Caesalpina spinosa*), gall nuts (pathological excrescences) from *Quercus infectoria* (Turkish gall) and *Rhus semialata* (Chinese gall), sumac leaves (*Rhus coriara*)	wood of oak (*Quercus* spp.), chestnut (*Castanea* spp.) and myrobalan (*Terminalia chebula*)	tropical shrub legumes, tea leaves	commonly found in fruits and seeds such as grapes, apple, olives, beans, sorghum grains, carob pods, cocoa, and coffee, besides tree bark and heart wood
common types				quebracho tannins from wood of *Schinopsis* spp., *Loxopterygium* spp. wattle tannins from *Acacia* spp. bark tannins from pine (*Pinus* spp.), oak (*Quercus* spp.), and gaboon wood (*Aucoumea klenaena*)

(*Schinopsis lorentzii* and *Schinopsis balansae*), and various tree barks.

The most abundant and well-studied flavonoids are based on the flavan-3-ol structure of (−)-epicatechin and (+)-catechin. Addition of a third phenolic group on the B ring yields epigallocatechin and gallocatechin. Flavan-3-ols with a single phenolic group on the B ring are less common. Figure 3 shows the structures of most common procyanidins.[13]

A group that occupies an intermediate position in the tannin hierarchy is the family of catechin tannins combining elements of hydrolyzable and condensed tannins. These tannins are quite common in tropical shrub legumes[14] and tea leaves.[15] Recently, gallic acid esters of proanthocyanidins have also been discovered.[16]

TANNIN CHARACTERIZATION

Both characterization and analysis of tannins are difficult due to their low solubility in organic solvents and their complex structures. They often occur in complex mixtures difficult to standardize and quantify. A variety of indirect and somewhat elusive analytical methods are currently available for the analysis of tannins, and they are based on the evaluation of their total phenolic hydroxyl group content or on the overall condensed or hydrolyzable tannin content as determined by specific functional group assays or on protein precipitable methods.[17−21] Because different phenolic groups give different responses, the "tannin level" or "phenolic level" of a sample cannot be adequately expressed as a single value. Another major limitation lies in the difficulty of preparing appropriate standards. Different responses prevent the use of a single commercially available compound as a convenient standard, because the relative responses of the standard and the analyte in the assay are not known. To overcome these difficulties, several methods based on different chemistries should be employed in parallel to obtain a qualitative and quantitative picture of the tannins present in the mixture.

The phenolic group assay methods are based on redox chemistry and include the Prussian blue and the Folin method, as well as other related procedures.[22,23] Differences in the redox potential and stoichiometry for different phenolics yield differential responses. Polyphenolics have different responses on molar mass analysis with respect to the standard gallic acid. For this reason the results are expressed as gallic acid equivalents rather than absolute weight percent.

Flavonoid monomers of condensed tannins can be quantified by functional group methods. The interflavanoid bond can be oxidatively cleaved by HCl in butanol, releasing proanthocyanidin that can be in turn quantified (but not specifically characterized).[24] The chemical characteristics of the tannins such as the regiochemistry of the interflavan bond or the oxygenation pattern affect the yields significantly. As an example, the yields reported for quebracho tannins are much lower than those for sorghum tannin, because the interflavan bond in quebracho is not quantitatively oxidized under the assay conditions. An alternative method, the vanillin method, is hardly reproducible and difficult to standardize.

Gallotannins can be quantified by hydrolysis followed by quantification of the released gallic acid by the rhodanine assay or the potassium iodate method.[25] Ellagitannins can be quantified by the quantification of ellagic acid released upon hydrolysis by the nitrous acid method.[26]

Tannin samples are often complex mixtures. To date, no characterization/quantification method that can afford purity,

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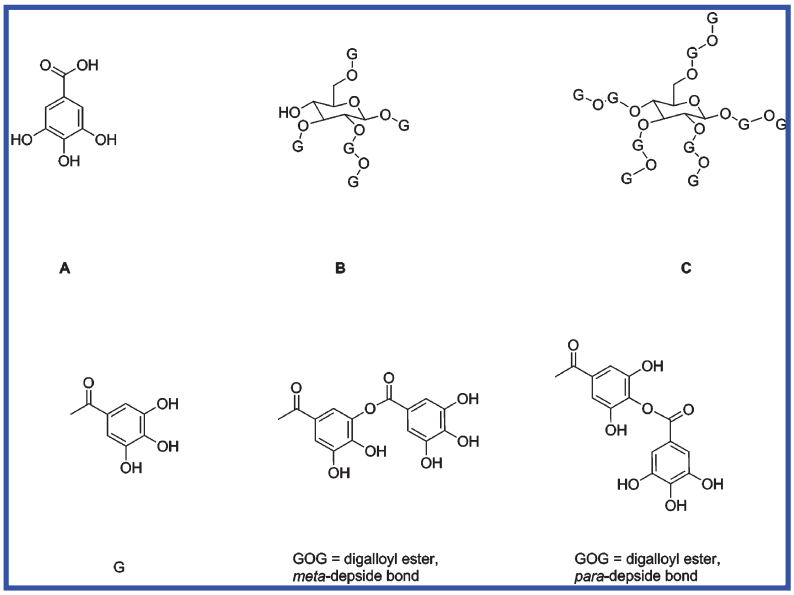

Figure 1. (A) PGG, (B) PGG isomers, and (C) nominal structure for tannic acid.

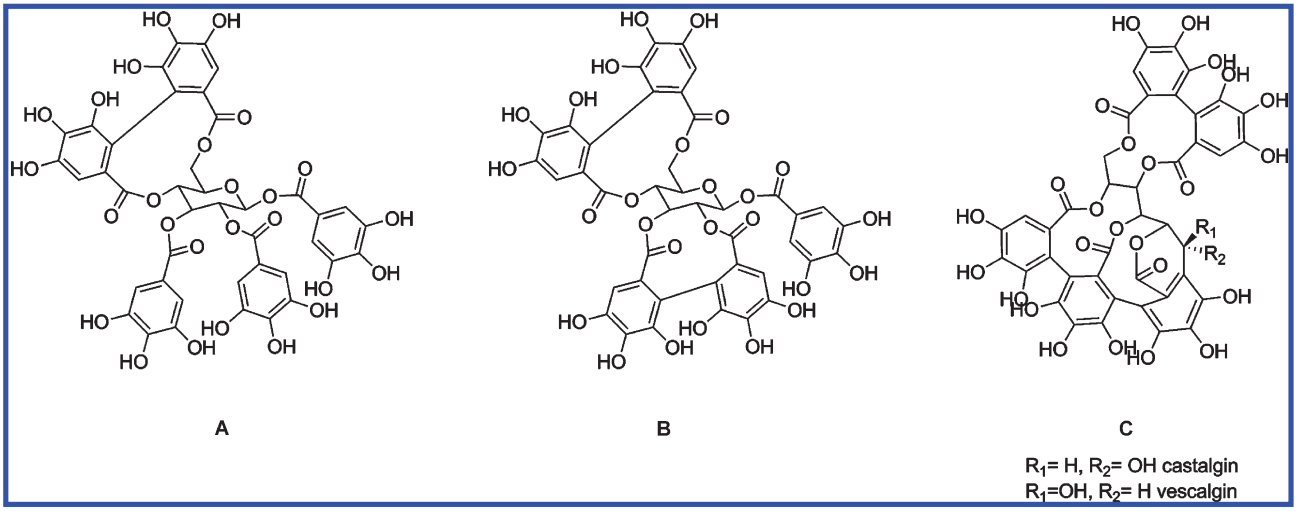

Figure 2. Ellagitannins: (A) eugeniin; (B) casuarictin; (C) castalgin (R_1 = H, R_2 = OH) and vescalgin (R_1 = OH, R_2 = H).

structural, and quantitative evaluation of a specific tannin sample is available. In all cases the structural characterization of complex mixtures cannot be performed unless careful chromatographic separation of the mixtures is carried out prior to further analyses.[27]

In principle, structural differences in tannins can be better evaluated by taking into consideration the aromatic ring substitution patterns that are present as phenolic, catecholic, ortho-substituted and ortho-disubstituted phenolic groups.

The early primary objective of our work was to develop a simple and reliable quantitative technique for tannin analysis, which would provide structural information on all of the fundamental functional groups present in them, namely, the phenolic hydroxyls and the carboxylic and aliphatic hydroxyl substructures. There are many examples of magnetic resonance techniques, when applied to polyphenolic polymers, proven to be excellent analytical tools for the structural elucidation of these complex biopolymers.[28−32] Accordingly, the work of our laboratory has been focused on the development of novel solution state ^{31}P-based NMR methods aimed at expanding the frontiers of applying NMR-based analysis methods to the analysis of tannins. We report here the development of a new ^{31}P hetronuclear correlated NMR spectroscopic technique applied to the elucidation of tannin structure. This solution state NMR technique is capable of detecting and quantitatively evaluating all functional groups in tannins possessing reactive hydroxyl groups, that is, aliphatic OH, phenolic OH, and carboxylic acid substructures after their selective and quantitative functionalization with a suitable phosphitylating reagent.

With the aim of developing a new analytical technique for the structural elucidation and quantitative evaluation of tannins from different sources, an array of different tannins model compounds was selected and submitted to ^{31}P NMR spectroscopic analysis. This allowed the definition of specific and well-resolved chemical shift regions for different tannin substructures.

MATERIALS AND METHODS

^{31}P NMR Analysis and Preparation NMR Solution. A solvent mixture of pyridine and $CDCl_3$ (1.6:1 v/v) was prepared under anhydrous conditions. Cholesterol was used as internal standard at a

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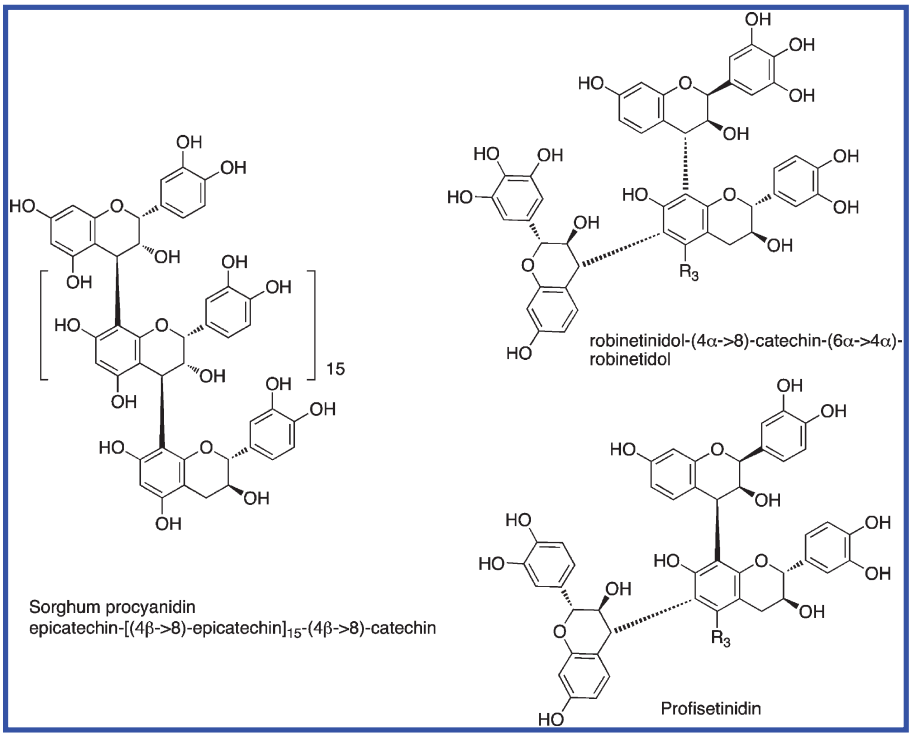

Figure 3. Procyanidins.

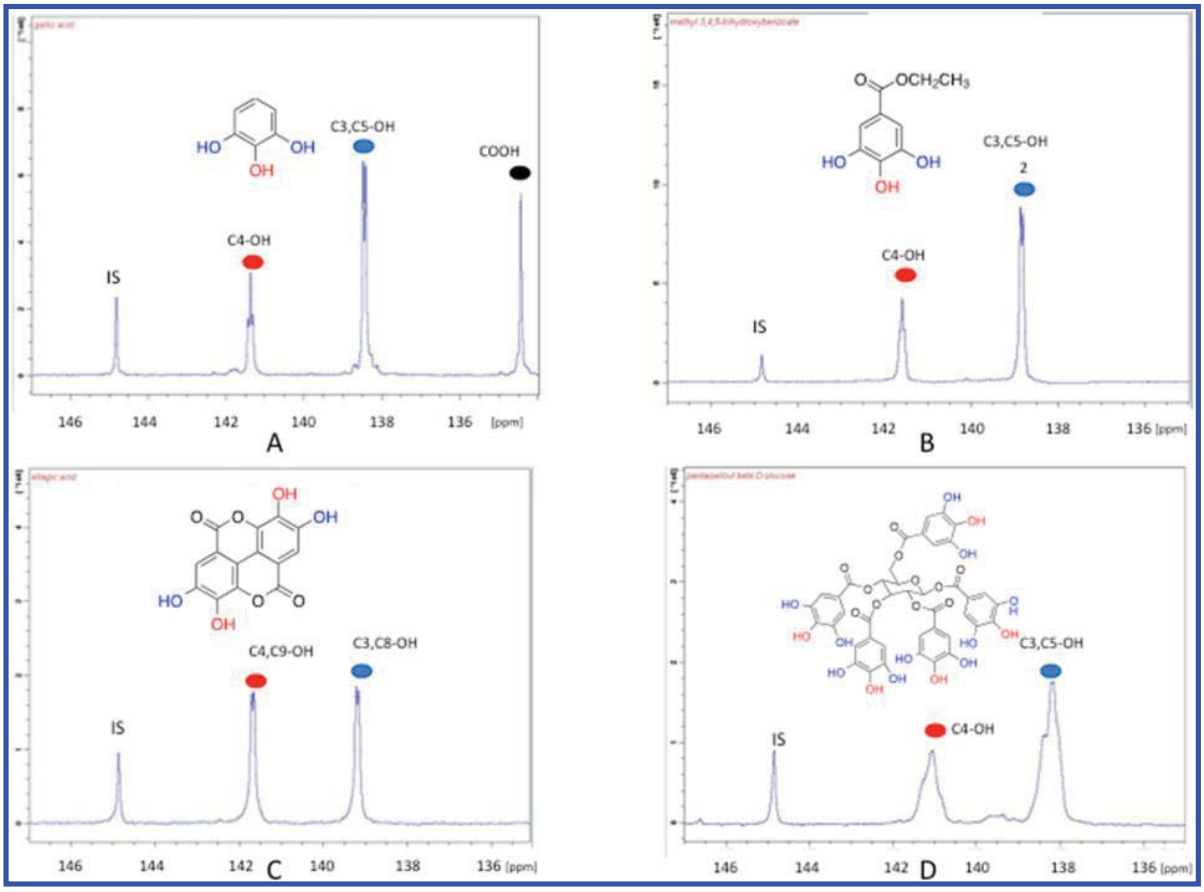

Figure 4. ^{31}P NMR spectra of hydrolyzable tannin model compounds phosphitylated with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (Cl-TMDP): (A) gallic acid; (B) methyl gallate; (C) ellagic acid; (D) pentagalloyl D-glucose.

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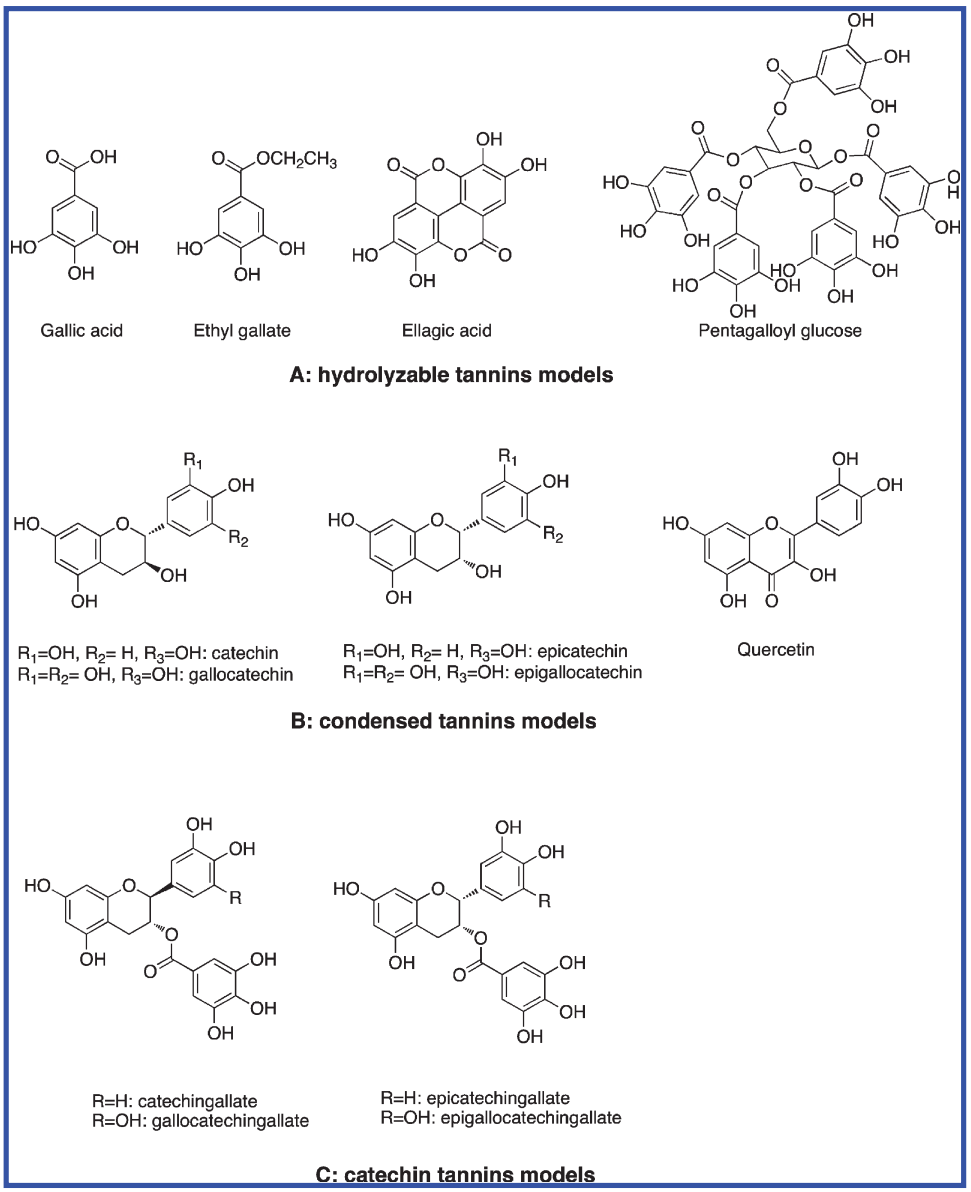
Chart 1. Tannins Model Compounds



concentration of 0.1 mol/L in the aforementioned NMR solvent mixture. Fifty milligrams of Cr(III) acetylacetonate was added as relaxation agent to this standard solution. The NMR solvent mixture was stored over molecular sieves (4Å) under an argon atmosphere.

Phosphitylation Procedure. Fifteen milligrams of the tannin model was accurately weighed in a volumetric flask and suspended in 400 μL of the solvent solution. One hundred microliters of the internal standard solution was added, followed by 100 μL of 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (Cl-TMDP). The flask was tightly closed, and the mixture was stirred for 90 min at ambient temperature.

NMR Spectroscopy. The ^{31}P NMR spectra were recorded on a Bruker 300 MHz NMR spectrometer. The probe temperature was set to 20 °C. To eliminate NOE effects, the inverse gated decoupling technique was used. Typical spectral parameters for quantitative studies were as follows: 90° pulse width and sweep width of 6600 Hz. The spectra were accumulated with a delay of 15 s between successive pulses. Line broadening of 4 Hz was applied, and a drift correction was performed prior to Fourier transform. Chemical shifts were expressed in parts per million from 85% H_3PO_4 as an external reference. All chemical shifts reported are relative to the reaction product of water with Cl-TMDP, which has been observed to give a sharp signal in pyridine/$CDCl_3$ at 132.2 ppm.

The spin−lattice relaxation profiles of the phosphorus atoms attached on tannins ranged between 2 and 0.5 s. The spin−lattice relaxation time of cholesterol was found to be 1.5 s. As such, the experimental protocol for spectral acquisition of tannins phosphitylated with Cl-TMDP was developed with a pulse delay of 15 s. All NMR experiments were carried out in triplicate. The standard deviation was found never to be higher than 0.2 mmol/g.

RESULTS

Phosphitylation Reactions. The reactions of various phospholane chlorides with labile centers present in coal and lignin samples were extensively investigated by Verkade's[33] and Argyropoulos's[28−30] research groups, respectively. The phospholane with particular potential for our primary objectives was that of Cl-TMDP. Cl-TMDP has been reported to react quantitatively with aliphatic alcohols, phenols, and carboxylic acids and polyphenols in pyridine.[29,30] Its reaction with labile protons proceeds as shown in Scheme 1. The fact that three

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Table 2. ^{31}P NMR Data of Tannin Model Compounds with Assignments and Respective Chemical Shifts

entry[a]	sample	aliphatic OH	*o*- disubstituted phenolic OH	*o*-substituted phenolic OH	*o*-unsubst phenolic OH	COOH
1	gallic acid		141.36	138.44		134.45
	methyl gallate		141.59	138.84		
	ellagic acid		141.67	139.17		
	pentagalloyl glucose		141.06	138.21		
2	catechin	145.29		139.00	137.65	
				138,87		
				138.07		
	epicatechin	145.94		139.44−139.37	137.67	
				138.89−138.83		
				137.89		
	gallocatechin	145.25	142.33	138.32	137.67	
				138.10		
	epigallocatechin	145.82	142.46	138.7	137.72	
				137.95		
	quercetin		141.92	140.60	137.40	136.50 (highly conjugated *β*-oxo phenol)
				138.50		
3	catechin gallate		141.41	138.43	137.72	
				138.42−138.80		
				137.72		
	epicatechin gallate		141.15	139.19−139.16	137.70	
				138.42−138.47		
				137.78		
				137.69		
	gallocatechin gallate		141.47	138.37−138.32	137.71	
			141.87	138.07−138.00		
				137.71		
	epigallocatechin gallate		141.99	138.66		
			141.17	137.85		
				137.65−137.59		

[a]Entry 1, hydrolyzable tannin model compounds; entry 2, condensed tannin model compounds; entry 3, catechin tannin model compounds.

oxygens surround the phosphorus atom in the phosphite esters formed ensures that the ^{31}P NMR signals of such derivatives will normally be singlets containing no coupling information.

The phosphitylation reaction was carried out in pyridine/$CDCl_3$ = 1.6:1. The choice of pyridine is due to the necessity to neutralize the hydrochloric acid that arises from the derivatization reaction as shown in Scheme 1.

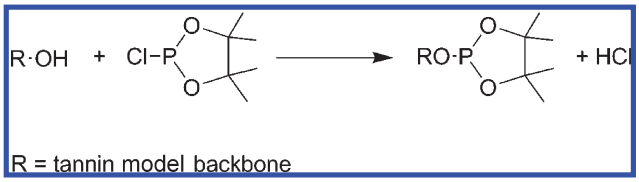
To quantitatively evaluate the amount of different functional groups, a suitable internal standard is needed. In this respect the choice was cholesterol. This compound has a chemical shift of 144.82 ppm when derivatized with Cl-TMDP and does not overlap with other tannin functional group signals. There are, however, suitable alternative standards that may also be used at will if desired so.

Quantitative ^{31}P NMR of Tannin Model Compounds. A wide array of hydrolyzable, condensed, and catechin tannin model compounds were selected and studied in detail. Chart 1 reports their structures.

The extent of functionalization of the different tannin functional groups was evaluated by reaction of Cl-TMDP with the selected model compounds. All of the model compounds were exactly weighed and submitted to ^{31}P labeling in the presence of precisely known amounts of internal standard. Comparative integrations of the NMR peaks due to the different functional groups with the one from the internal standard showed that in all cases the derivatization of the tannin models was quantitative.

Hydrolyzable Tannin Model Compounds. The simplest class of tannins consists of hydrolyzable tannins. Gallic acid, ethyl gallate, ellagic acid, and pentagalloyl glucose (Chart 1A) were selected as representative hydrolyzable tannin model compounds. Their quantitative ^{31}P NMR spectra after phosphitylation are shown in Figure 4, panels A, B, C, and D, respectively.

Scheme 1. Phosphitylation of Tannin Model Compounds with 2-Chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (Cl-TMDP)



Gallic acid (Figure 4A) shows three signals: the first is attributable to the ortho-disubstituted phenolic OH at 141.36 ppm; the second, which accounts for two catecholic signals, shows double intensity at 138.44 ppm; and the third, due to the carboxylic moiety, was apparent at 134.45 ppm. A long-range ^{31}P coupling was evident because the downfield peak was a triplet, whereas the catecholic signal was a doublet. The absorbance regions were found to be in accordance with previous assignment

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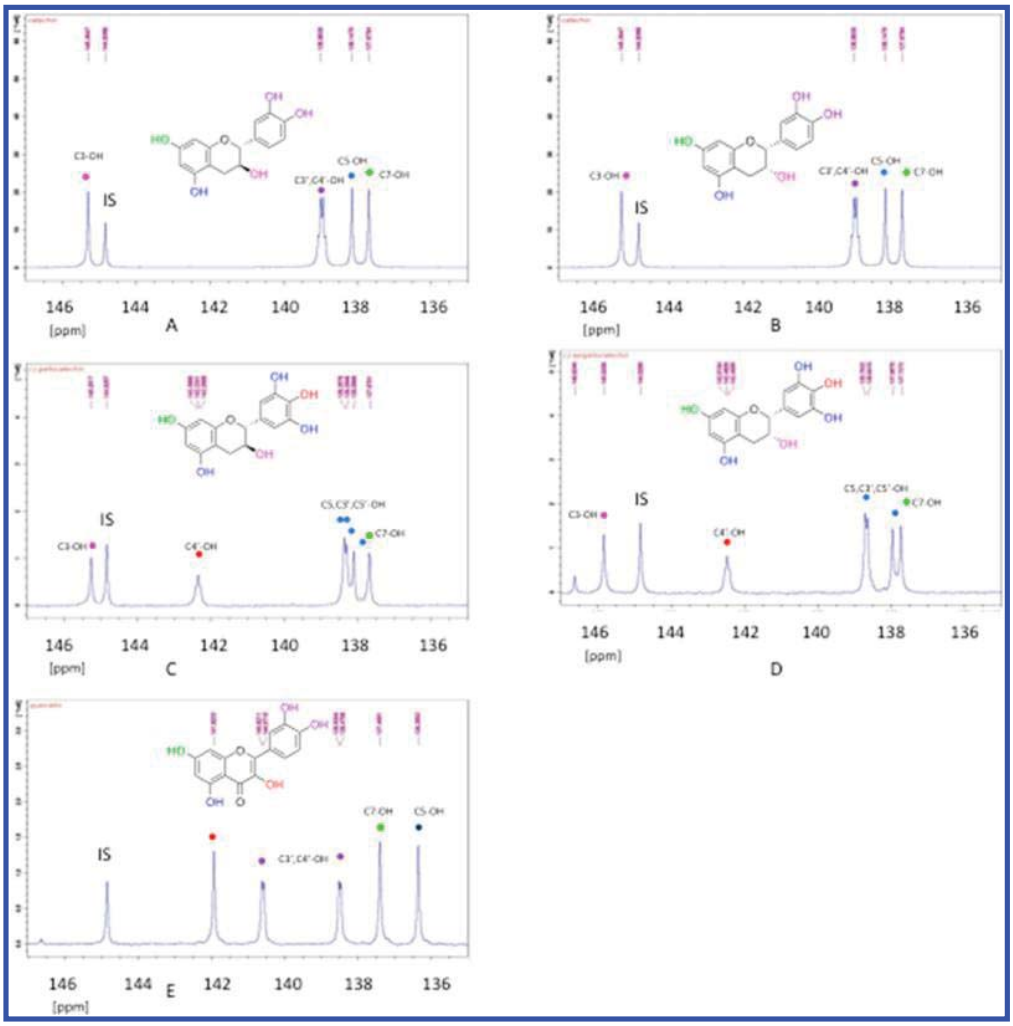

Figure 5. ^{31}P NMR spectra of proanthocyanidin model compounds phosphitylated with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (Cl-TMDP): (A) catechin; (B) epicatechin; (C) gallocatechin; (D) epigallocatechin; (E) quercetin.

of different phenolic and carboxylic moieties of lignin model compounds previously studied.[28−30] In the same fashion ethyl gallate showed the disubstituted phenolic OH absorbance at 141.59 ppm and the catecholic signals at 138.84 ppm. As expected in this case a carboxylic acid signal was missing (Figure 4B). Ellagic acid showed only two signals of equal intensity assigned to the ortho-disubstituted, 141.67, and the catecholic, 139.17 ppm, phenolics, respectively (Figure 4C). In both cases the presence of a rigid structure deshielded the signals with respect to those of gallic acid.

The more complex pentagalloyl glucose showed two sets of signals attributed to the ortho-disubstituted phenolic at 141.06 ppm and to the two catecholic moieties per gallate residue, 138.21 ppm, respectively. Esterification of the polyol led to an upfield all of the phenolic signals by about 0.5 ppm with respect to those of the free acid. The absence of carboxylic and aliphatic OH absorbances shows the purity of the sample that is both completely esterified and does not contain free gallic acid. Table 2, entry 1, shows the specific signal assignments.

Condensed Tannin Model Compounds. Catechin, epicatechin, gallocatechin, epigallocatechin, and quercetin (structures shown in Chart 1B) were used as representative compounds for condensed tannin substructures. The quantitative ^{31}P NMR spectra of the phosphitylated models (Figure 5, panels A, B, C, D, and E, respectively) showed an interesting behavior revealing actual stereochemical information. In fact, the more crowded epicatechin and epigallocatechin showed downfield signals with respect to the corresponding catechin homologues (Table 2, entry 2). Quercetin is not precisely a tannin model, because it presents the flavanol ring oxidized to the corresponding flavonol. This structure is of high interest because oxidized proanthocyanidin substructures are easily formed in condensed tannins. The spectrum contains five different signals due to the catecholic OH groups (positions 3′ and 4′ of ring B, respectively) the ortho-disubstituted OH in position 3 of ring C, and the ortho-unsubstituted OH in position 7 of the A ring. The keto group on the flavonol moiety introduced hydrogen bonding with the phenolic position on the C5 with possible formation of a stable six-membered ring. In this case the phosphitylation of the C5 phenolic group resulted in an upfield signal as shown in Figure 5E (Table 2, entry 2).

Catechin Tannin Model Compounds. The NMR spectra of the other models with flavonoid−gallate structures are shown with the respective structures in Chart 1C. The study of the spectra allowed the assignment of signals due to gallate and flavonoid structures, respectively (Table 2, entry 3). In these cases all of the phenolic positions and the aliphatic OH groups were found to be quantitatively phosphitylated. Figure 6 shows

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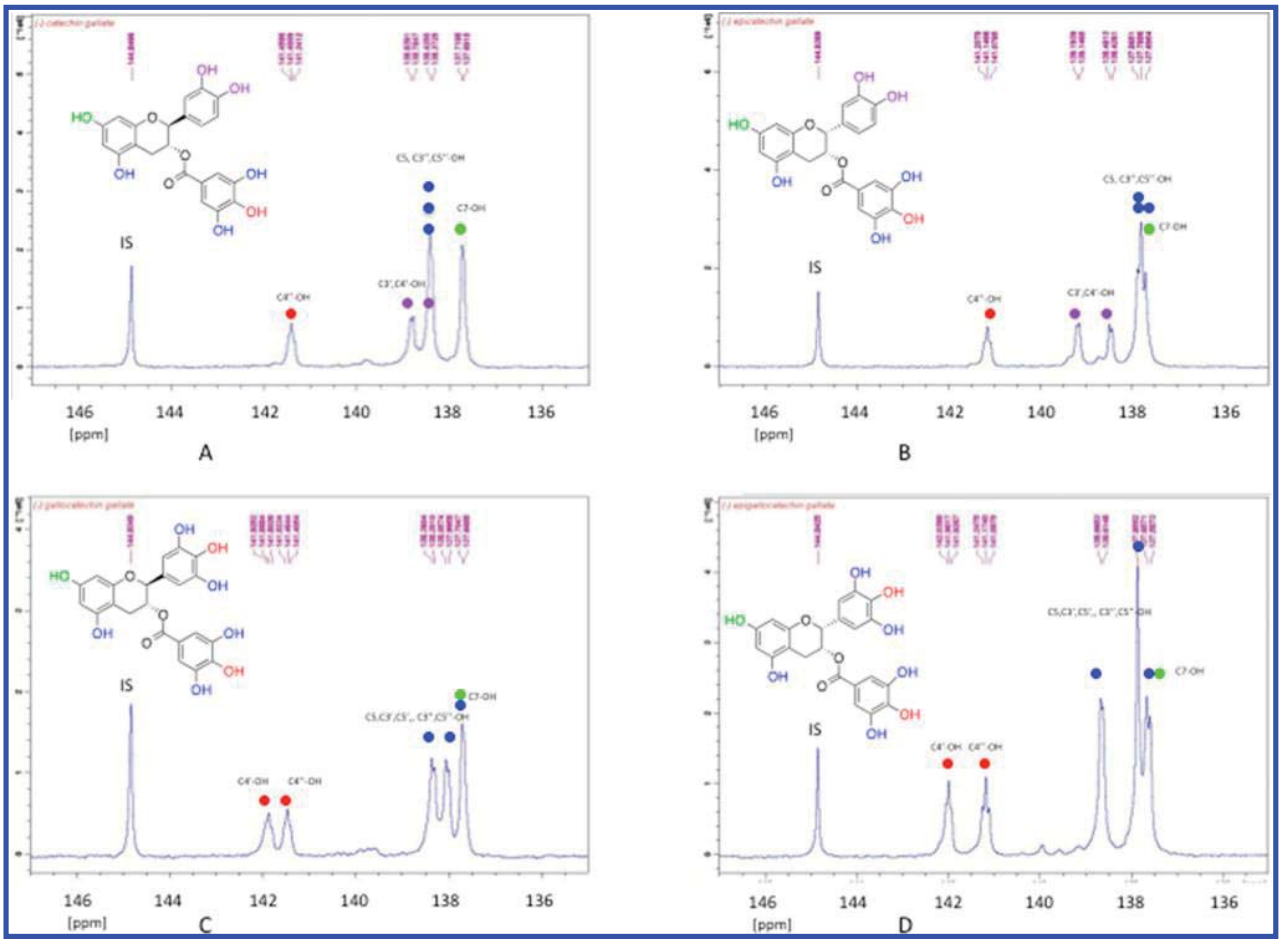

Figure 6. ^{31}P NMR spectra of cathechin tannin model compounds phosphitylated with 2-chloro-4,4,5,5-tetramethyl-1-1,3,2-dioxaphospholane (Cl-TMDP): (A) catechin gallate; (B) epicatechin gallate; (C) gallocatechin gallate; (D) epigallocatechin gallate.

the ^{31}P NMR spectra. Table 2, entry 3, shows their structural assignments.

Table 3 reports the chemical shifts of different functional groups in tannin model compounds derivatized with Cl-TMDP and submitted to quantitative ^{31}P NMR. It is interesting to note that the chemical shifts of different phenolic groups are clearly distinct. More specifically, the ortho-disubstituted, the ortho-substituted, and the ortho-unsubstituted phenolic moieties present different absorption ranges that can be readily identified. More specifically, according to the family of compounds considered, it is also possible to assign separately catecholic moieties.

This would allow the definition of specific ranges of chemical shifts typical of each aliphatic phenolic and carboxylic moiety present in tannin samples.

In summary, quantitative in situ phosphorus labeling of an array of condensed, hydrolyzable, and catechin tannin model compounds was accomplished, and the ^{31}P NMR spectra of the suitably in situ phosphitylated compounds were recorded. The different ^{31}P-labeled aliphatic, phenolic OH groups, and carboxylic acids showed specific and distinguished regions of absorbance. The presence of a suitable internal standard allowed the evaluation of the extent of labeling and the establishment of the completeness of the reactions. All of the labile OH groups were quantitatively functionalized,

This solution NMR technique is the first reported analytical method capable of simultaneously detecting and quantitatively evaluating all functional groups in tannins and flavonoids possessing reactive hydroxyl groups, that is, aliphatic OH, phenolic OH, and carboxylic acid substructures. As such, it represents an invaluable analytical tool for the detection and quantification of these elusive and biologically active polyphenolic compounds.

Table 3. ^{31}P NMR Signal Assignments and Chemical Shift of Tannin Model Compounds Derivatized with Cl-TMDP

signal	chemical shift (ppm)	
aliphatic OH	145.94−145.25	
ortho-disubstituted OH	142.46−141.06	141.06−141.47 gallate 142.46−141.87 gallo/epigallocatechin
ortho-substituted (*o*-phenol) OH	140.60−137.59	140.2−138.3 catechols 138.8−137.6 noncatechols
ortho-unsubstituted OH	137.72−137.40	
COOH	135.5−134.0	
total phenolic OH	144.0−137.0	

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Notes

The authors declare no competing financial interest.

REFERENCES

(1) Crestini, C.; Melone, F.; Sette, M.; Saladino, R. Milled wood lignin: a linear oligomer. *Biomacromolecules* **2011**, *12* (11), 3928−3935.

(2) Spencer, C. M.; Cai, Y.; Martin, R.; Gaffney, S. H.; Goulding, P. N.; Magnolato, D.; Lilley, T. H.; Haslam, E. Polyphenol complexation-some thoughts and obstarvation. *Phytochemistry* **1988**, *27*, 2397−2409.

(3) Hagerman, A. E.; Butler, L. G. The specificity of proanthocyanidine-protein intereaction. *J. Biol. Chem.* **1981**, *256*, 4494−4497.

(4) McLeod, M. N. Plant tannins: their role in forage quality. *Nutr. Abstr. Rev. (Ser. B)* **1974**, *44*, 803−815.

(5) Mueller-Hrvey, I.; McAllan, A. B. Tannins: their biochemistry and nutritional properties. *Adv. Plant Cell Biochem. Biotechnol.* **1992**, *1*, 151−217.

(6) Haslam, E. Plant polyphenols: vegetable tannins revisited. In *Chemistry and Pharmacology of Natural Products*; Phillipson, J. D, Ayres, D. C., Baxter, H., Eds.; Cambridge University Press: Cambridge, UK, 1989; pp 1−230.

(7) Mole, S. The systematic distribution of tannins in the leaves of angiosperms: a tool for ecological studies. *Biochem. Syst. Ecol.* **1993**, *21*, 833−846.

(8) Hoste, H. The effects of tannin-rich plants on parasitic nematodes in ruminants. *Trends Parasitol.* **2006**, *22*, 253−261.

(9) Field, J. A.; Lettinga, G. Toxicity of tannic compounds to microorganisms. Plants polyphenols; synthesis, properties, significance. *Basic Life Sci.* **1992**, *59*, 673−692.

(10) Nonaka, G. Isolation and structure elucidation of tannins. *Pure Appl. Chem.* **1989**, *61*, 357−360.

(11) Yoshida, T. Chemistry and function of vegetable polyphenols with high molecular weights. *BioFactors* **2000**, *13*, 121−125.

(12) Lei, Z. *Monomeric Ellagitannins in Oak and Sweetgum*. Ph.D. thesis, Virginia Polytechnic Institute and State University, Blacksburg, VA, 2002.

(13) Fine, A. M. Oligomeric proanthocyanidin complexes: history, structure, and phytopharmaceutical applications. *Altern. Med. Rev.* **2000**, *5*, 144−151.

(14) Mueller-Harvey, I.; Reed, J. D.; Hartley, R. D. Characterisation of phenolic compounds, including flavonoids and tannins, of ten Ethiopian browse species by high performance liquid chromatography. *J. Sci. Food Agric.* **1987**, *39*, 1−14.

(15) Graham, H. N. Green tea composition, consumption, and polyphenol chemistry. *Preventive Med.* **1992**, *21*, 334−350.

(16) Porter, L. J. Flavans and proanthocyanidins. In *The Flavonoids Advances in Research Since 1986*; Harborne, J. B., Ed.; Chapman and Hall: London, UK, 1994; pp 23−55.

(17) Kiessoun, K.; Souza, A. Polyphenol contents, antioxidant and anti-inflammatory activities of six malvaceae species traditionally used to treat hepatitis B in Burkina Faso. *Eur. J. Sci. Res.* **2010**, *44*, 570−580.

(18) Ramamoorthy, P. K.; Bono, A. Antioxidant activity, total phenolic and flavonoid content of *Morinda citrifolia* fruit extracts from various extraction processes. *J. Eng. Sci. Technol.* **2007**, *2*, 70−80.

(19) Tahir, P. M.; Musgrave, O. C.; Ashaari, Z. Determination of polyphenolic content of barks extracts for wood adhesives. *Holzforshung* **2002**, *56*, 267−272.

(20) Hagerman, A. E.; Butler, L. G. Protein precipitation method for the quantitative determination of tannins. *J. Agric. Food Chem.* **1978**, *26*, 809−812.

(21) *Quantification of Tannins in Tree Foliage*; FAO/IAEA Working Document; IAEA: Vienna, Austria, 2000.

(22) Vernon, L.; Singleton, R. O.; Lamuela-Raventós, R. M. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods Enzymol.* **1999**, *299*, 152−178.

(23) Price, M. P.; Butler, L. G. Rapid visual estimation and spectrophotometric determination of tannin content of sorghum grain. *J. Agric. Food Chem.* **1977**, *25*, 1268−1273.

(24) Porter, L. J.; Hrstich, L. N.; Chan, B. G. The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. *Phytochemistry* **1986**, *25*, 223−230.

(25) Inoue, K. H.; Hagerman, A. E. Determination of gallotannin with rhodanine. *Anal. Biochem.* **1988**, *169*, 363−369.

(26) Bate-Smith, E. C. Detection and determination of ellagitannins. *Phytochemistry* **1972**, *11*, 1153−1156.

(27) Strumeyer, D. H.; Malin, M. J. Condensed tannins in grain sorghum: isolation, fractionation, and characterization. *J. Agric. Food Chem.* **1975**, *23*, 909−914.

(28) Granata, A.; Argyropoulos, D. S. 2-Chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane, a reagent for the accurate determination of the uncondensed and condensed phenolic moieties in lignins. *J. Agric. Food Chem.* **1995**, *43*, 1538−1544.

(29) Argyropoulos, D. S. ^{31}P-NMR in wood chemistry: a review of recent progress. *Res. Chem. Intermed.* **1995**, *21*, 373−395.

(30) Jiang, Z. H.; Argyropoulos, D. S.; Granata, A. Correlation analysis of phosphorus-31 NMR chemical shifts with substituent effect of phenols. *Magn. Reson. Chem.* **1995**, *33*, 375−382.

(31) Ahvazi, B. C.; Crestini, C.; Argyropoulos, D. S. ^{19}F nuclear magnetic resonance spectroscopy for the quantitative detection and classification of carbonyl groups in lignins. *J. Agric. Food Chem.* **1999**, *47*, 190−201.

(32) Crestini, C.; Argyropoulos, D. S. Structural analysis of wheat straw lignin by quantitative ^{31}P and 2D NMR spectroscopy. The occurrence of ester bonds and α-O-4 substructures. *J. Agric. Food Chem.* **1997**, *45*, 1212−1219.

(33) Schiff, D. E.; Verkade, J. G.; Metzler, R. M.; Squires, T. G.; Venier, C. G. Determination of alcohols, phenols, and carboxylic acids using phosphorus-31 NMR spectroscopy. *Appl. Spectrosc.* **1986**, *40*, 348−351.