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# Tannin Structural Elucidation and Quantitative $^{31}$P NMR Analysis. 2. Hydrolyzable Tannins and Proanthocyanidins

Federica Melone,[†,§] Raffaele Saladino,[§] Heiko Lange,[†] and Claudia Crestini*[,†]

[†]Department of Chemical Sciences and Technologies, University of Rome 'Tor Vergata', Via della Ricerca Scientifica, 00133 Rome, Italy

[§]Department of Ecology and Biological Sciences, Tuscia University, Via San Camillo de Lellis, 01100 Viterbo, Italy

**ABSTRACT:** An unprecedented analytical method that allows simultaneous structural and quantitative characterization of all functional groups present in tannins is reported. In situ labeling of all labile H groups (aliphatic and phenolic hydroxyls and carboxylic acids) with a phosphorus-containing reagent (Cl-TMDP) followed by quantitative $^{31}$P NMR acquisition constitutes a novel fast and reliable analytical tool for the analysis of tannins and proanthocyanidins with significant implications for the fields of food and feed analyses, tannery, and the development of natural polyphenolics containing products.

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

## INTRODUCTION

Tannins are naturally occurring plant polyphenols. Their main characteristic is that they bind proteins, basic compounds, pigments, large molecular weight compounds, and metallic ions and display antioxidant activities.[1−3] They are present in fruits (grapes, persimmon, blueberry, etc.), in tea, in chocolate, in coffee, in legume forages (trefoil, etc.), in legumes, in trees (*Acacia* spp., *Sesbania* spp., etc.), and in grasses (sorghum, corn, etc.).[4,5]

Hydrolyzable tannins are galloyl esters and their derivatives, in which galloyl moieties or their derivatives are attached to a variety of polyol, catechin, and triterpenoid cores (gallotannins, ellagitannins, and complex tannins) (Figure 1). The simplest hydrolyzable proto-tannin is pentagalloyl glucose (*β*-1,2,3,4,6-pentagalloyl-*O*-D-glucopyranose) (PGG) (Figure 1B).[6] Tannic acid is described as glucose pentagalloylgallate, but it is actually a mixture of different isomers and partially galloylated glucose (Figure 1C). Commercial sources for tannic acid are poorly defined in composition. It has to be noted that PGG is the core ester of Chinese or sumac gallotannin, whereas Turkish gallotannin is composed of esters of 1,2,3,6-tetragalloyl glucose or 1,3,4,6-tetragalloyl glucose. Several species of maple (*Acer pseudoplatanus*, etc.) contain glucitol and several chestnut species (*Castanea* sp.) and northern red oak (*Quercus rubra*) contain hammamellose as polyol core. Hydrolysis with strong acids converts gallotannins to gallic acid and the core polyol.[7]

Ellagitannins are esters of hexahydrodiphenic acid (HHDP) (Figure 2) that in turn is generated by oxidative cross-linking of two galloyl groups.[8]

Condensed tannins are oligomeric and polymeric proanthocyanidins that can possess different interflavanyl coupling and substitution patterns. They are commonly found in fruits and seeds such as grapes, apples, olives, beans, sorghum grains, carob pods, cocoa, and coffee, as well as in tree bark and heartwood.[9]

The most abundant and best studied procyanidins are based on the flavan-3-ol structure of (−)-epicatechin and (+)-catechin (Figure 3).[10] Addition of a third phenolic group on the B ring yields epigallocatechin and gallocatechin, whereas flavan-3-ols with a single phenolic group on the B ring are less common (Figure 3).[11] The flavonol units are linked mainly via a carbon−carbon bond between the C-8 of the terminal unit and the C-4 of the extender as, for example, in sorghum procyanidin. Linear polymers based on C-4/C-6 linkages and branched dimers with both C-4/C-6 and C-4/C-8 linkages are less common. An important group of proanthocyanidins are the 5-deoxyflavan-3-ol polymers (Figure 3). Branching is common in these tannins because of the reactivity of the deoxy A ring as in prorobinetinedins and profisetinidins. They comprise the major tannins in quebracho and acacia tannin preparations.

Tropical shrub legumes and tea leaves[12] are rich in catechin tannins that combine the flavonoid and gallic moieties.[13]

## TANNIN CHARACTERIZATION

The extensive presence of tannins in a variety of foods, possessing many variable structures, creates formidable analytical challenges. These challenges are applicable to both isolated tannins and tannins in complex matrices.

The biological activities displayed by tannins are due to the variable amounts and regiochemical details of their phenolic OH groups, which actually regulate their protein-binding capacities and their antioxidant activities.

As such, the specific characterization of the regiochemical patterns of the phenolic groups in tannins and their quantification represent an invaluable tool for the study of their characteristics and the evaluation of quality and activity of a widespread array of foods, feeds, and nutraceutical products. However, the characterization and analysis of tannins are difficult due to their complex structure and low solubility in organic solvents. They often occur in complex mixtures difficult to standardize and quantify. The most widely used methods of

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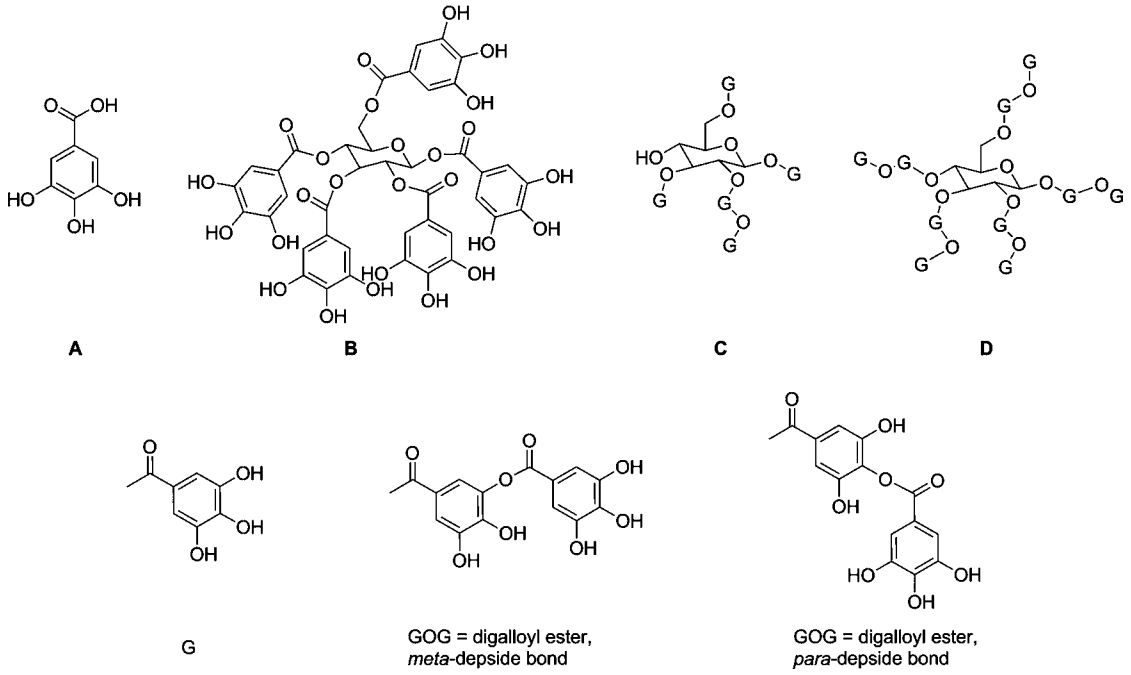


**Figure 1.** (A) Gallic acid, (B) PGG, (C) PGG isomers, and (D) nominal structure for tannic acid.

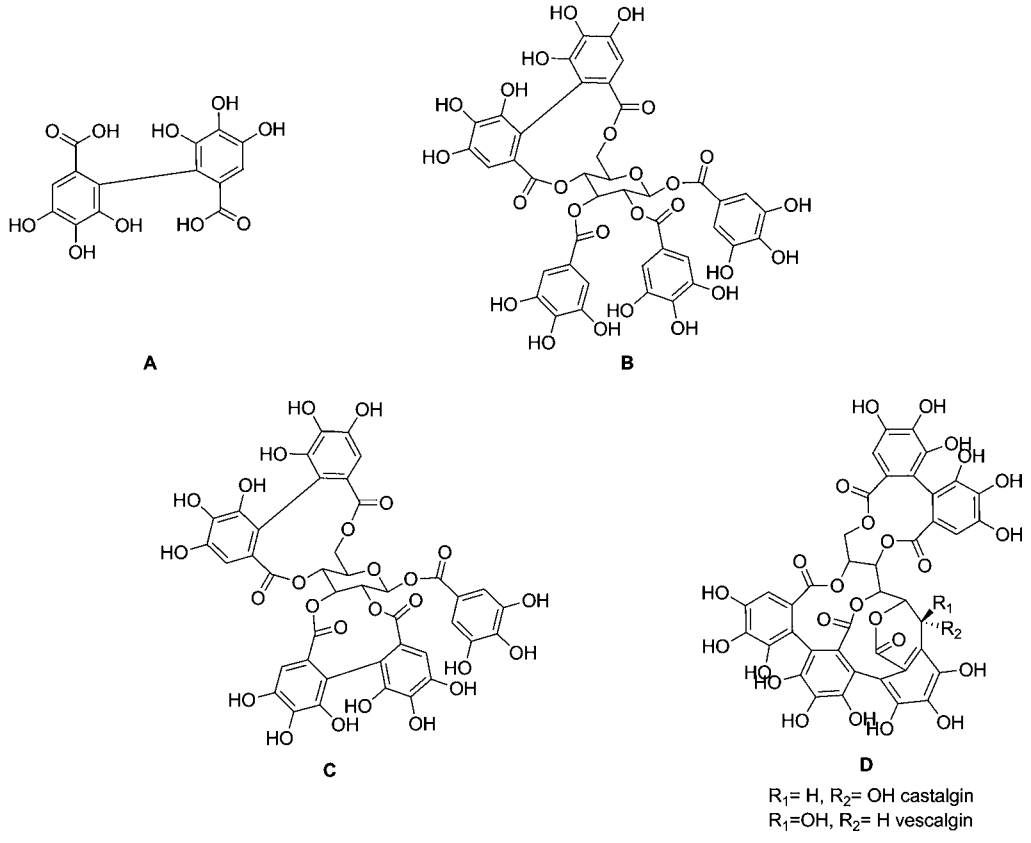


**Figure 2.** (A) Hexahydrodiphenic acid (HHDP), (B) eugeniin, (C) casuarictin, and (D) castalgin ($R_1$ = H, $R_2$ = OH) and vescalgin ($R_1$ = OH, $R_2$ = H).

analysis are based on the general determination of the phenolic group content,[14,15] on the overall condensed or hydrolyzable tannin content (using specific functional group assays), and on protein precipitable methods.[16] Because different phenolic groups give different responses to such methods of analysis, the "tannin level" or "phenolic level" of a sample cannot be adequately expressed as a single value. Another major limitation common to all methods of analysis lies in the difficulty of preparing appropriate standards. Varying responses observed for different tannins prevent the use of a single commercially available compound as a convenient standard, because the


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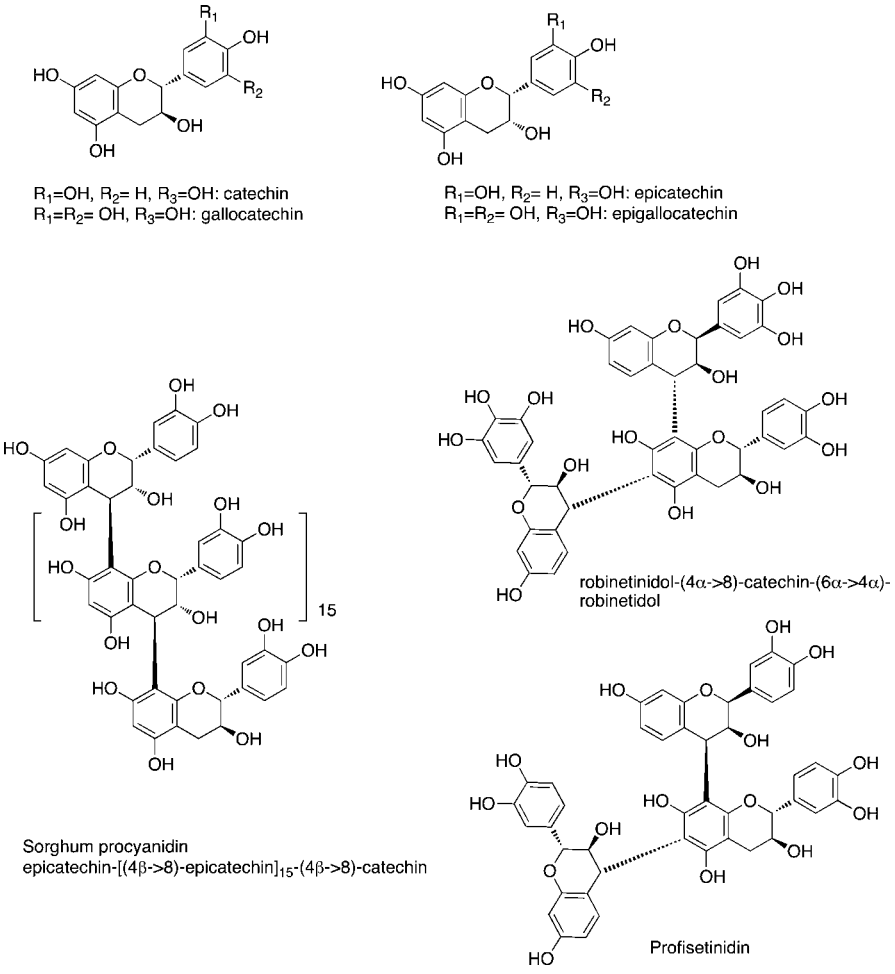


**Figure 3.** Procyanidins.

relative responses of the standard and the sample in the assay are not known.[17−21]

Structural differences in tannins and their biological activities can be better evaluated by taking into consideration the aromatic ring substitution patterns that present phenolic, catecholic, ortho-substituted, and ortho-disubstituted phenolic groups, because these moieties are responsible for the metal binding and antioxidant properties of tannins. The aim of our work was the development of a new simple and reliable quantitative analytical technique for the analysis of tannins that would be able to structurally characterize and quantitatively evaluate different classes of tannins not only in isolated tannin samples but also in complex mixtures such as fruit juices, wines, foods, feeds, and plant extracts. This requires a versatile and flexible analytical protocol that is able to provide information on the fundamental functional structural features present within the tannin sample, namely, the phenolic, the carboxylic, and the aliphatic hydroxyl groups.

There are many examples for the application of magnetic resonance techniques in the analyses of polyphenolic polymers, all demonstrating that these techniques are excellent analytical tools for the structural elucidation of complex biopolymers.[22−35] Accordingly, the work in our laboratory has been focused on the development of novel solution state $^{31}$P nuclear magnetic resonance methods aimed to expand the frontiers of application of NMR to tannin analysis. We recently reported the characterization of a wide array of tannin model compounds.[36] It was possible to identify specific regions of absorbance of labeled ortho-disubstituted, ortho-monosubstituted, and ortho-unsubstituted phenolic groups, respectively, besides aliphatic OH groups and carboxylic acids. In this effort we have embarked on the identification and quantitative evaluation of the different structural features for an array of hydrolyzable tannins and proanthocyanidins. The study of different tannin substructures by this new technique allowed for the first time further delving into and clarification of the composition of complex tannin preparations and the evaluation of their purity based on an a $^{31}$P NMR fingerprint output. Notably, otherwise insoluble tannins become well solubilized in the pyridine/$CDCl_3$ NMR solvent mixture we use for phosphitylation.

## EXPERIMENTAL PROCEDURES

**Tannin Isolation.** Tannin isolation was performed as previously described in archival literature.[37,38] Wood chips were defatted by shaking with diethyl ether. The dried defatted wood was then treated with 30% aqueous acetone using a Soxhlet extractor overnight. The extractive was finally concentrated under reduced pressure and freeze-dried.

**Tannin Purification.** Tannin purification was performed as previously described in the archival literature.[38] About 100 mg of the dried extractive was purified on a column of Sephadex LH-20 equilibrated with ethanol. The sample was dissolved in a sufficient aliquot of ethanol (a drop of water is eventurally necessary to achieve complete solubilization), loaded on the column, and eluted with

ethanol thoroughly. The ethanol-soluble fraction was collected and concentrated under reduced pressure. The column was then eluted with 50% aqueous acetone. The collected tannin fraction was concentrated and freeze-dried.

**Analysis of the Total Phenolic and Polyphenolic Antioxidants (Folin−Ciocalteu Assay).**[14] *Calibration Curve.* Five hundred milligrams of gallic acid was dissolved in 10 mL of ethanol and diluted with water in a 100 mL volumetric flask. Solutions of gallic acid at 0, 50, 100, 150, 250, and 500 mg/L were prepared by dissolving 0, 1, 2, 3, 5, and 10 mL of the previous stock solution into a 100 mL volumetric flask and diluting to volume with water. Twenty microliters of each gallic acid solution was pipetted into a cuvette containing 1580 μL of water; then 100 μL of the Folin−Ciocalteu reagent was added. After 2 min, 300 μL of a sodium carbonate solution (1 g/10 mL) was added. The mixtures were stirred at 40 °C for 30 min before the absorbance was read at 765 nm against the blank for each solution. The calibration curve was obtained plotting the absorbances versus the concentrations.

*Assay.* Solutions of 50 mg/L of different tannins were prepared. As for the calibration curve, 20 μL of each sample was pipetted into a cuvette containing 1580 μL of water. After the addition of 100 μL of Folin−Ciocalteu reagent and 300 μL of the sodium carbonate solution, the reaction mixture was allowed to stir at 40 °C for 30 min. The absorbance was recorded at 765 nm against the blank. Results are reported in gallic acid equivalents (GAE).

**GPC Analysis.** Tannin (7−10 mg) was suspended in 2 mL of a mixture of acetic acid/acetyl bromide 92:8 v/v and stirred at room temperature during 2 h. The solvent was evaporated under reduced pressure, and the residue was dissolved in 5 mL of THF. Twenty microliters was injected into a Shimadzu LC 20AT liquid chromatograph equipped with two GPC/SEC columns connected in series (Agilent PL gel 5 μm, 500 Å, followed by Agilent PL gel 5 μm, 1000 Å) and with an SPD M20A ultraviolet diode array (UV) detector. The analysis was carried out using THF as eluent at a flow rate of 0.50 mL/min, and the absorbance was recorded at 280 nm. The GPC system has been calibrated against polystyrene standards (molecular weight range of 580−1.95 × $10^6$ g/mol).

**$^{31}$P NMR Analysis.** *Preparation of NMR Solution.* A solvent mixture of pyridine and $CDCl_3$ (1.6:1 v/v) was prepared under anhydrous conditions. The internal standard solution was prepared using cholesterol at a concentration of 38.67 mg/mL (0.1 M) in the above-mentioned 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.* Seven to ten milligrams of tannin were accurately weighed in a volumetric flask and suspended in 300 μL of the NMR solvent mixture. One hundred microliters of the 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 at 20 °C for 90 min, rendering the initially heterogeneous mixture homogeneous.

*NMR Spectroscopy.* The $^{31}$P NMR spectra were recorded on a Bruker 300 MHz 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, 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.[22,23,32,36]

The spin−lattice relaxation profiles of the phosphorus atoms attached to tannin backbones range from 2 to 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 did not exceed 0.2 mmol/g. This accounts for a high reproducibility guaranteed by this analytical method. An issue could rise in signal integration. The phasing of the region of interest should be carried out carefully. Usually baseline correction is not needed nor integral phasing. However, in case of baseline correction the integrals phases should be corrected as well.

## RESULTS

**Phosphitylation Reactions.** The reactions of various phospholane chlorides with labile centers present in coal and lignin samples were investigated by Verkade's[33] and Argyropoulos's[39−41] research groups, respectively. Recently, we reported the use of 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (Cl-TMDP) for the structural characterization and quantitative evaluation of tannin model compounds. Cl-TMDP reacts quantitatively with aliphatic alcohols, phenols, and carboxylic acid moieties of tannin models.[36] The fact that three oxygen atoms surround the phosphorus atom in the phosphite esters formed ensures that the $^{31}$P NMR signals of such derivatives will appear as singlets containing no coupling information, with exception of the long-range coupling occurring in catecholic moieties.

The phosphitylation reaction was carried out in pyridine and chloroform 1.6:1 as per our effort described for the tannin model compounds.[36]

To quantitatively evaluate the amount of different functional groups a suitable internal standard is needed. There are several compounds used as internal standards in quantitative $^{31}$P NMR spectroscopy. With the aim of evaluating the best choice for tannin analysis, we compared the degree of functionalization of different potential standards: cholesterol, *N*-hydroxynaphthalimide, and endo-*N*-hydroxy-5-norbornene-2,3-dicarboximide were taken into consideration as possible standards. An equimolar solution of the three standards was prepared, reacted with Cl-TMDP for 2 h at ambient temperature, and analyzed after 2 h. The integral areas of the three peaks were found to be different, as expected, with *N*-hydroxynaphthalimide and endo-*N*-hydroxy-5-norbornene-2,3-dicarboximide showing a significantly smaller area than cholesterol. This means that the overall amount of OH groups determined by quantitative $^{31}$P NMR in the presence of *N*-hydroxynaphthalimide or endo-*N*-hydroxy-5-norbornene-2,3-dicarboximide is overestimated due to incomplete or unstable functionalization of the standards. On the contrary, cholesterol was found to be quantitatively labeled (data not shown) and thus selected as standard. Phosphitylated cholesterol has a chemical shift of 144.82 ppm when derivatized with Cl-TMDP and does not overlap with other tannin functional groups.

The analysis of tannin model compounds (see part 1[36]) allowed the definition of specific ranges of chemical shift typical for aliphatic, phenolic, and carboxylic moieties present in a tannin sample. Table 1 reports the signal assignment regions.

**Tannin Analysis.** An array of commercial gallotannins, ellagitannins, and proanthocyanidins was selected. Tannins were also extracted from *Eucalyptus grandis* bark, *Arundo donax* wood, and *Vitis vinifera* wood according to literature standard procedures to compare laboratory preparations with commercial samples.

**Gallotannins.** A sample of tannic acid from Chinese natural gall nuts was submitted to quantitative $^{31}$P NMR after phosphitylation in situ with Cl-TMDP (Figure 4A). Signals present in the acid region clearly indicate that the sample contains free acids, and among them gallic acid. It is also possible to identify two regions of signals attributable to ortho-

**Table 1. Signal Assignment of $^{31}$P NMR Spectra of Tannins Labeled with 2-Chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane Based on the Analysis of Tannin Model Compounds**

| 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 OH (*o*-phenol) | 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 | |

disubstituted and ortho-substituted phenolics, ranging from 142.2 to 141.00 ppm and from 139.5 to 138.0 ppm, respectively.

The disubstituted phenolics show two distinct peaks. According to the terminal gallate chemical shift shown by pentagalloyl glucose, it is possible to assign the signal at 141.2 ppm to the terminal gallates and the signal at 141.9 ppm to the internal ortho-disubstituted phenolics. The ortho-substituted phenols are grouped into two distinct signal clusters centered at 139.1 and 138.4 ppm, respectively. The integrals of the single peak cluster allow further insight into the tannin structure. In fact, the ratio of the ortho-disubstituted phenolics provides quantitative information about the regiochemistry of the depside bond. More specifically, in case the tannin contains a meta-depside regiochemistry, the amounts of terminal and internal disubstituted phenolics are the same. In a fully meta-depside array, one would anticipate five internal and five terminal disubstituted OH groups per molecule. In contrast, when para-depside bonds are present, the internal ortho-disubstituted phenolics are not present in the tannin. The integrals showed a 4:5 ratio between the internal and external ortho-disubstituted bonds. This implies that one para-depside bond is present for every five terminal gallate units, implying that there is on average one para-depside bond per tannin molecule. This is also confirmed by the integration in the ortho-substituted region. In fact, in this case we determined the presence of 16 phenolic OH groups, in precise accordance with the presence of one para-depside bonding pattern per tannic acid molecule.

When we turned our attention to the analysis of a commercial tannin sample from a different origin extracted from Turkish oak natural gall nuts (Figure 4B), a completely different $^{31}$P NMR spectrum was obtained, indicative of the discerning power of the proposed methodology proposed here. It was possible to identify two clusters of signals. One of them due to the terminal ortho-disubstituted phenolics appears at a chemical shift (141.3 ppm) that is within the range of chemical shifts (141.1−141.8 ppm) that were identified for a sample of gallic acid derivatives[36] (Figure 4B). The deshielded signal cluster was attributed to internal ortho-disubstituted phenolics, because steric hindrance leads to deshielding of the $^{31}$P NMR nuclei of phosphitylated phenols.[23] Furthermore, it was possible to identify and quantify the signals due to the ortho-substituted phenolics. An aliphatic OH group is present, thus indicating that PGG is not the core of this extract. The integration of the signals showed that this tannin is fundamentally a tetragalloylgallate and that the core polyol presents one free aliphatic OH group. The integration of the two ortho-disubstituted signals showed a 3.5:4.0 ratio. This indicates the occurrence of one para-depside bond for every eight galloylgallate units, meaning that the average frequency of the para-depside bond is one for every two tannin molecules. This is confirmed by the integration of the signals in the region of ortho-substituted phenolics, which sums to a value of 8.5.

It is also possible to determine the purity of the sample. Besides the presence of aliphatic OH signals that indicate partial esterification of the core polyol (as, for example, in chestnut gallotannins), the presence of acid signals indicates the presence of free gallic acid as a sign of tannin degradation. Significant amounts of gallic acid are also present in the Turkish oak gall sample as shown by the signal in the acidic region (134.5 ppm).

The GPC analysis of the samples (Figure 5) indicates the presence of an array of products with different molecular weights, thus confirming the heterogeneous nature of tannic acid preparations.

**Ellagitannins.** Commercial chestnut and oak wood ellagitannins were also subjected to quantitative $^{31}$P NMR analyses (Figure 6, panels A and B, respectively). The quantitative $^{31}$P NMR spectra of the two commercial ellagitannins were more complex than those of the gallotannins. Intense aliphatic OH signals were apparent. This indicates the presence of carbohydrates in the sample. The phenolic region

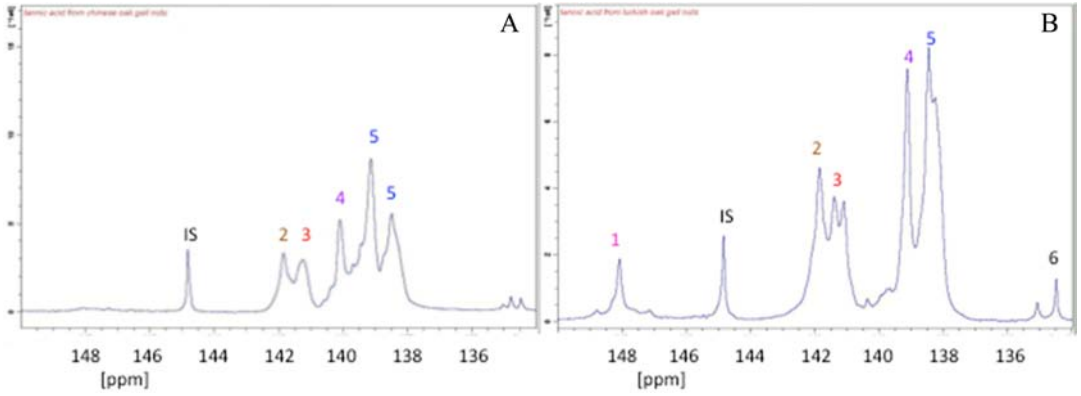


**Figure 4.** $^{31}$P NMR of tannic acid extracted from natural Chinese oak gall (A) and natural Turkish oak gall (B) after phosphitylation with Cl-TMDP. Signal assignment: 1, aliphatic OH; 2, ortho-disubstituted phenol, internal gallate; 3, ortho-disubstituted phenol, terminal gallate; 4, catechol; 5, ortho-substituted phenol; 6, COOH. Ranges of signal assignment are reported in Table 1.


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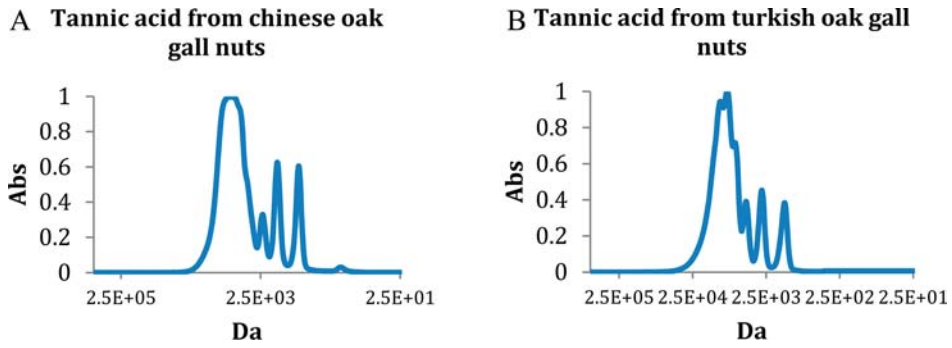


**Figure 5.** GPC analysis of tannic acid extracted from natural Chinese oak gall (A) and natural Turkish oak gall (B).

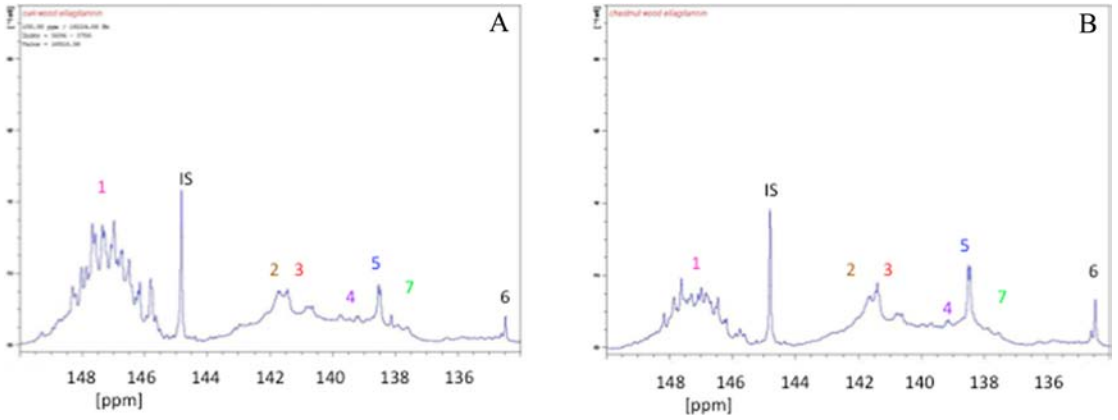


**Figure 6.** $^{31}$P NMR of ellagitannin extracted from chestnut (A) and oak wood (B) after phosphitylation with Cl-TMDP. Signal assignment: 1, aliphatic OH; 2, 3, ortho-disubstituted phenol; 4, 5, ortho-substituted phenol; 7, ortho-unsubstituted phenol; 6, COOH. Ranges of signal assignments are reported in Table 1.

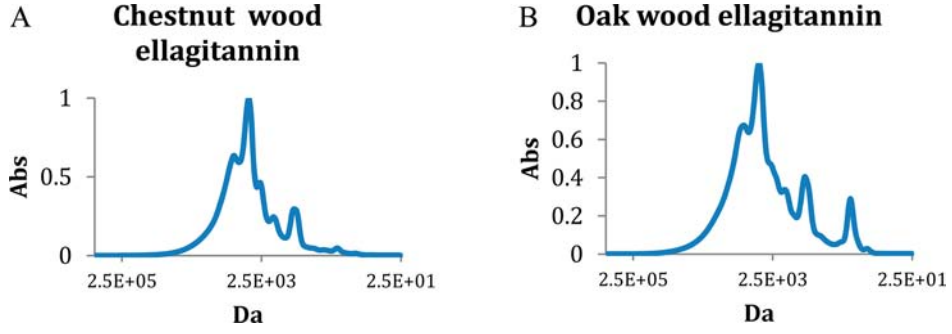


**Figure 7.** GPC of ellagitannin extracted from chestnut (A) and oak wood (B).

showed broad absorbance in the regions of the ortho-disubstituted, catecholic, and ortho-substituted phenolics. This is in agreement with the highly complex ellagitannin structures reported in the literature.[8] Despite this, both spectra show markedly similar fingerprints showing maximum peaks at 141.4, 138.4, and 134.4 ppm, which are typical for gallate residues, and at 141.6 ppm typical of the ellagic ortho-disubstituted phenolics. The relative amount of aliphatic OH groups is a criterion for the evaluation of the tannin purity. Furthermore, ellagitannins from different origins can be characterized by the ration of the ortho-disubstituted to ortho-monosubstituted phenolic groups and by the COOH content. This provides an invaluable analytical handle for the unequivocal characterization of ellagitannins. The GPC profiles of two different ellagitannins are shown in Figure 7; thus they show similar molecular weight distributions and thus matching the impression raised by the comparison of the NMR fingerprint.

**Proanthocyanidins.** The third class of tannins considered in this effort was that of condensed tannins. As such, tannin extracted from *V. vinifera* wood was purified, analyzed, and compared to a commercial grape seed tannin.

Commercial grape seed tannin displays a particular quantitative $^{31}$P NMR spectrum where signals characteristic for catechols, as well as ortho-substituted and ortho-unsubstituted phenolic groups were present, originating from the catechin and epicatechin substructures. The absence of ortho-disubstituted phenolic groups is noteworthy. This can be explained only on the basis of a polymerization between the flavonol units involving C4 and C8. In fact, a C4/C6 polymerization would imply the presence of ortho-disubstituted OH groups (Figure 2). A significant cluster of aliphatic OH


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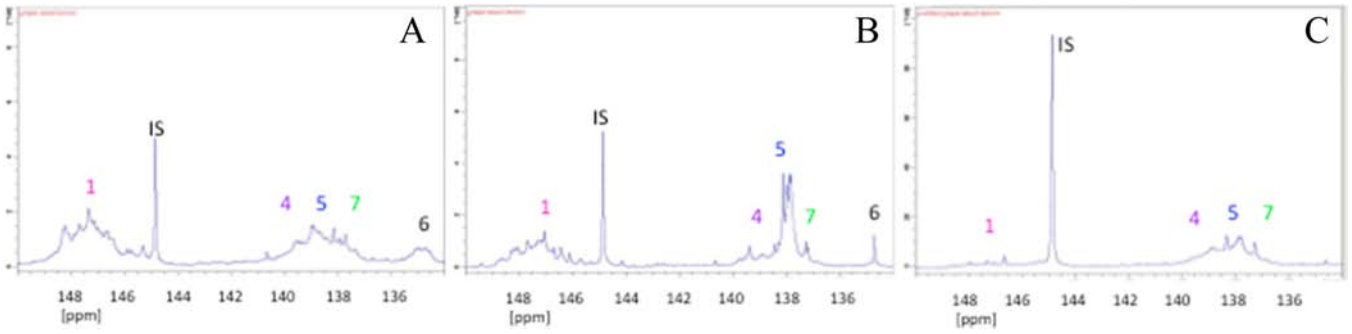


**Figure 8.** $^{31}$P NMR of tannin extracted from grape seeds (A) and grape wood (B) and purified grape wood tannin (C) after phosphitylation with Cl-TMDP. Signal assignment: 1, aliphatic OH; 4, 5, ortho-substituted phenol; 7, ortho-unsubstituted phenol; 6, COOH. Ranges of signal assignment are reported in Table 1.

groups is also present in the sample. This spectrum is basically analogous to those obtained for tannins extracted from grape wood (Figure 8A,B). To understand if the aliphatic OH signals are due to glycosylated tannins or to a contamination of the tannin sample, grape tannin was submitted to purification by chromatography on Sephadex. Figure 8C shows the spectrum of the tannin after purification. It is clear that the carbohydrate fraction is effectively removed by purification, and as such it is not chemically bound to the tannin, but indeed is an impurity. Figure 9 shows the GPC chromatograms of grape wood tannin

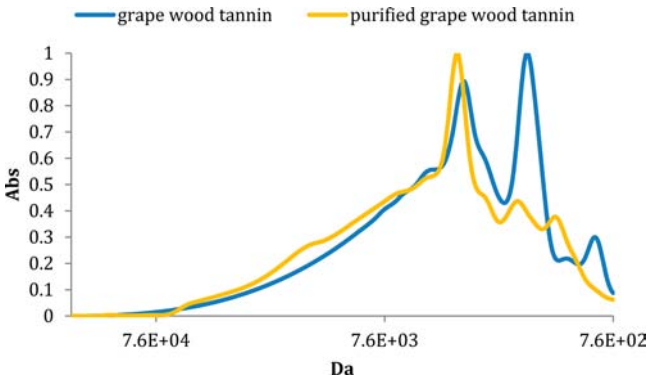


**Figure 9.** SEC of grape wood tannin before and after purification.

before and after purification. After purification, the high molecular weight profile was not significantly altered, whereas relatively low molecular weight fractions were removed.

**Tannin Total Phenolic Content.** To compare the newly developed method for analysis of tannins with previous analytical techniques, the total phenolic content of the tannins studied was evaluated. More specifically, integrating the quantitative NMR spectra of $^{31}$P-labeled samples, allows to arrive at a precise total phenolic content expressed in millimoles per 100 mg of sample. This could then be compared with the traditional Folin−Ciocalteu method expressed in milligrams gallic acid equivalents (GAE) per 100 mg of sample.[42] Table 2 shows the actual data obtained by the two methods. Although the general trend of total phenolic groups content is confirmed, the two methods do not correlate well ($R^2$ = 0.79). This is due to the fact that the Folin−Ciocalteu method expresses the results in GAE rather than by the actual total amount of phenolic groups. This does not make much sense in the case of proanthocyanidins, because they are not structurally related to gallic acid. In fact, as it has been demonstrated that the phosphitylation and subsequent quantitative $^{31}$P NMR spectroscopy is indeed quantitative for a wide array of tannin model compounds, this should be considered as being the more reliable method, especially in view of the fact that the response to the Folin−Ciocalteu method is not linear for all tannins.[42]

**Table 2. Comparison of the Total Phenolic Content of Tannin Samples As Evaluated by the Folin−Ciocalteu Method and by $^{31}$P NMR of Samples Phosphitylated with Cl-TMDP**

| tannin sample | mg GAE/100 mg[a] | phenolic OH (mmol/100 mg)[b] |
|---|---|---|
| gallic acid | 100 | 160 |
| ellagic acid | 101 | 132 |
| tannic acid from Chinese oak gall | 74 | 147 |
| tannic acid from Turkish gall nut | 111 | 200 |
| chestnut ellagitannin | 76 | 85 |
| oak wood ellagitannin | 63 | 55 |
| grape seeds tannin | 69 | 48 |

[a]Milligrams of gallic acid equivalent per 100 mg of tannin sample. [b]Millimoles of phenolic OH per 100 mg of tannin sample.

In addition to the quantification of the total phenolic groups, the quantitative $^{31}$P NMR analysis of labeled samples allows the facile and detailed quantification of all different classes of phenolic groups, the ortho-disubstituted, the ortho-substituted, and the ortho-unsubstituted ones. This allows the detailed characterization of pure tannins or mixtures of tannin samples.

The facile and detailed quantification of the aliphatic OH groups and of the carboxylic acids present in a given tannin sample allows for the evaluation of the sample's purity. Table 3 compares the different aliphatic, phenolic OH, and carboxylic acid contents of the different samples studied.

Recapitulating, the in situ phosphorus labeling of gallotannins, ellagitannins, and proanthocyanidins was accomplished. The quantitative $^{31}$P NMR spectra of suitably phosphitylated tannins were recorded. The different $^{31}$P-labeled aliphatic, phenolic OH groups, and carboxylic acids showed specific and distinguished regions of absorbance, assigned on the basis of the corresponding signals displayed by model compounds. The presence of a suitable internal standard allowed the facile quantification of these groups.

Comparison of the quantitative $^{31}$P NMR spectra of tannins from different origins shows specific distribution of differently substituted phenolic hydroxyl groups (Figures 4, 6, and 8). As such, quantitative $^{31}$P NMR makes it possible to establish the nature of a tannin sample as gallic, ellagic, or proanthocyanidin.


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Table 3. Aliphatic, Phenolic OH, and Carboxylic Acid Contents of the Different Tannin Samples Studied As Evaluated by $^{31}P$ NMR of Samples Phosphitylated in Situ with Cl-TMDP

| sample/functional group (mmol/g) | aliphatic OH (mmol/g) | ortho-disubstituted OH (mmol/g) | ortho-substituted OH (mmol/g) | ortho-unsubstituted OH (mmol/g) | COOH (mmol/g) |
|---|---|---|---|---|---|
| gallic acid | | 5.35 | 10.80 | | 5.36 |
| ellagic acid | | 6.62 | 6.63 | | |
| Chinese oak gall tannic acid | | 5.29 | 9.40 | | 0.31 |
| Turkish oak gall tannic acid | 0.96 | 5.40 | 9.15 | | 0.32 |
| chestnut wood ellagitannin | 4.41 | 3.60 | 1.44 | 1.76 | 0.45 |
| oak wood ellagitannin | 5.89 | 2.58 | 1.00 | 0.97 | 0.27 |
| grape seeds tannin | 4.34 | 0.33 | 0.87 | 2.21 | 0.56 |
| grape wood tannin | 2.11 | 0.12 | 0.50 | 2.22 | 0.16 |
| purified grape wood tannin | 0.75 | 0.72 | 1.24 | 3.76 | |

Beyond this, each tannin sample offers a specific fingerprint that can be used in principle for quality control, namely, its classification and purity evaluation. In the case of gallotannins it is possible to unambiguously assign the amount and regiochemistry of esterification. Consequently, the presented solution NMR technique allows for the unprecedented quantitative and qualitative structural elucidation of gallo- and ellagitannins and proanthocyanidins. As such, it represents an invaluable analytical tool for the detection and quantification of such elusive and biologically active polyphenolic compounds.

## AUTHOR INFORMATION


### Corresponding Author

*(C.C.) E-mail: crestini@uniroma2.it.



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### Notes

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