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**^1H - ^{13}C HSQC NMR Spectroscopy for Estimating Procyanidin/Prodelphinidin and
Cis/Trans-Flavan-3-ol Ratios of Condensed Tannin Samples: Correlation with Thiolytic**

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1 **ABSTRACT:** Studies with a diverse array of 22 purified condensed tannin (CT) samples from
2 nine plant species demonstrated that procyanidin/prodelphinidin (PC/PD) and *cis/trans*-flavan-3-
3 ol ratios can be appraised by ^1H - ^{13}C HSQC NMR spectroscopy. The method was developed from
4 samples containing 44 to ~100% CT, PC/PD ratios ranging from 0/100 to 99/1, and *cis/trans*
5 ratios from 58/42 to 95/5 as determined by thiolysis with benzyl mercaptan. Integration of cross-
6 peak contours of H/C-6' signals from PC and of H/C-2',6' signals from PD yielded nuclei
7 adjusted estimates that were highly correlated with PC/PD ratios obtained by thiolysis ($R^2 =$
8 0.99). *Cis/trans*-flavan-3-ol ratios, obtained by integration of the respective H/C-4 cross-peak
9 contours, were also related to determinations made by thiolysis ($R^2 = 0.89$). Overall, ^1H - ^{13}C
10 HSQC NMR spectroscopy appears to be a viable alternative to thiolysis for estimating PC/PD
11 and *cis/trans* ratios of CT, if precautions are taken to avoid integration of cross-peak contours of
12 contaminants.

13 **KEYWORDS:** Condensed tannins, proanthocyanidins, procyanidins, prodelphinidins, nuclear
14 magnetic resonance spectroscopy, NMR, thiolysis

15

INTRODUCTION

16 Condensed tannins (CTs) (also referred to as proanthocyanidins or PACs) represent a class of
17 polyphenolic plant secondary metabolites that are composed of oligomers and polymers of
18 flavan-3-ols.^{1,2} These structures vary not only in flavan-3-ol subunit composition, but also in
19 interflavan-3-ol bond connectivity and mean degree of polymerization (mDP). Condensed
20 tannins are most commonly composed of procyanidin (PC) subunits derived from catechin and
21 epicatechin and of prodelphinidin (PD) subunits derived from gallo catechin and
22 epigallocatechin. Substituents at C-2 and C-3 in the C-ring of epicatechin and epigallocatechin
23 have a *cis* configuration while catechin and gallo catechin possess a *trans* stereochemical
24 orientation (Figure 1). These subunits are typically interconnected by C4-C8 interflavan-3-ol
25 linkages (classified as a B-type linkage, Figure 1), but other less common interunit linkages such
26 as the C4-C6 also occur in CTs.

27 A major point of interest in CTs stems from the potential positive impact they could bring
28 to the agricultural industry because of their ability to modulate proteolysis during forage
29 conservation and ruminal digestion,³⁻⁷ to prevent bloat,⁸ reduce intestinal parasite burdens⁹ and
30 lessen methane emissions from ruminants.^{10,11} It is thought that the CT composition may play a
31 role in how effectively they impart their biological effects on each of these outcomes, improving
32 both the economical and environmental sustainability of ruminant farm operations. Thus, results
33 from *in vitro* and *in vivo* experiments where CT content is known and the composition is well-
34 defined should reveal CT types and levels that are required for optimizing ruminant health and
35 productivity. Such information would help plant breeders with selection for CT content and
36 structure and also help identify plant varieties that are good candidates for genetic modification.

37 Analytical techniques allowing for the rapid assessment of chemical structures of CT
38 mixtures within and isolated from plant materials remain a high priority.¹² Development of
39 robust analytical methods is required to gain a better understanding of how CTs affect the
40 interdependency of CT/protein structure-activity relationships. Owing to the structural
41 complexity of CTs, novel approaches are needed for their analysis, including new techniques to
42 corroborate data from existing methods. These analytical techniques are needed for analyzing CT
43 mixtures as these are relevant, and applicable to, nutritional and health research on CTs for both
44 humans¹³ and animals.¹⁴

45 A variety of analytical techniques have been developed for the characterization and
46 analysis of condensed tannins. Thiolytic with benzyl mercaptan^{15,16} is one of the most common
47 methods to obtain compositional and structural data on *in situ* or isolated CT.¹⁷ This method
48 involves acid-catalyzed degradation of CT polymers into reactive monomeric cationic subunits
49 which are subsequently trapped with nucleophiles, such as benzyl mercaptan, providing stable
50 monomeric flavan-3-ol adducts. In this method, extension units are converted into stable C-4
51 thio ethers whereas terminal units of the polymers are liberated as intact flavan-3-ol monomers.
52 HPLC analysis of the mixtures obtained from these depolymerization studies allows qualitative
53 and quantitative assessment of CTs composition in terms of ratios of PC/PD and *cis/trans*
54 subunits and overall mDP. It can thus be used to calculate the purity of isolated CT samples
55 based on the total flavan-3-ol yield. Currently, thiolytic represents one of the most useful
56 techniques available for the analysis of CT composition.

57 One dimensional (1D) NMR spectroscopic studies have been used previously to
58 determine the compositional aspects of isolated condensed tannin samples by either solution state
59 ¹³C NMR spectroscopy¹⁸⁻²⁷ or cross-polarization magic angle spinning (CPMAS) solid state ¹³C

60 NMR spectroscopy.²⁸⁻³¹ Solution state ¹³C NMR spectroscopy has been utilized for
61 determination of PC/PD^{18-20,22-27} and *cis/trans* ratios,^{18,19,22,24-27} estimations of mDP^{18-20,22,}
62 ^{24,26,27} and the identification of C4-C6 and C4-C8 linkages.^{20,21} These NMR techniques, however,
63 suffer from broad and often times unresolved signals, long acquisition times, and low signal-to-
64 noise ratios which hamper an accurate assessment of CT composition. Solid phase studies of CT-
65 containing plant material have been conducted using ¹³C CPMAS NMR techniques.²⁸⁻³¹
66 Although this technique provides good signal-to-noise ratios, signals in the spectra are still broad
67 and frequently overlap with non-CT signals. In addition, ¹³C CPMAS requires the use of highly
68 specialized equipment.

69 By contrast, common two-dimensional (2D) NMR techniques have not been extensively
70 explored for assessing the composition of either purified CTs or CT present in whole plant
71 materials.³² Here we report the use of ¹H-¹³C HSQC NMR spectroscopy as a means to determine
72 PC/PD and *cis/trans* ratios of isolated CT samples.

73 MATERIALS AND METHODS

74 General Procedure for Purification and Characterization of Condensed Tannins.

75 Condensed tannins were purified from dried and milled plant material and analyzed for CT
76 composition and purity as previously described.^{15,16} Briefly, dried plant material was milled
77 (typically using a cyclone mill) containing a 1 or 0.5 mm screen and the resulting ground
78 material was extracted with 7:3 acetone/water (3 x 10 mL/g of dried material) and filtered. The
79 combined filtrates were concentrated on a rotary evaporator (<40 °C) to remove acetone and the
80 resulting aqueous layer was extracted with one-half volume of dichloromethane (2 x) and was
81 freeze-dried. The freeze-dried residue was purified in one of two ways. The first method

82 involved dissolving the freeze-dried residue in water and applying the resulting mixture to the
83 top of a Sephadex LH-20 column pre-packed in water. The column was eluted with water,
84 removing a majority of the carbohydrates present. Column elution was continued with 3:7
85 acetone/water (providing sample fraction 1) followed by elution of the column with 1:1
86 acetone/water to give sample fraction 2, which typically contained CTs of highest purity.
87 Alternatively, the dried extraction residue is adsorbed onto Sephadex LH-20 as a 1:1
88 methanol/water solution to provide a mixture with the consistency of wet sand. This material is
89 then placed in a Buchner funnel and consecutively rinsed with methanol/water (1:1) followed by
90 a series of acetone/water mixtures (1:1, 7:3, 9:1) with each rinsing conducted three times with a 5
91 mL solvent per gram of Sephadex LH-20. The three rinse filtrates for each solvent were pooled,
92 concentrated on a rotary evaporator (<40 °C) to remove the volatile solvent and freeze-dried. In
93 both purification methods, the freeze-dried samples were analyzed by ^1H - ^{13}C HSQC NMR
94 spectroscopy to assess relative purity and/or thiolysis to provide a numerical purity.

95 **NMR Spectroscopy.** ^1H , ^{13}C and ^1H - ^{13}C HSQC NMR spectra were recorded at 27 °C on a
96 BrukerBiospin DMX-500 (^1H 500.13 MHz, ^{13}C 125.76 MHz) instrument equipped with TopSpin
97 2.1 software and a cryogenically cooled 5-mm TXI $^1\text{H}/^{13}\text{C}/^{15}\text{N}$ gradient probe in inverse
98 geometry. Spectra were recorded in DMSO- d_6 /pyridine- d_5 (4:1) mixtures and were referenced to
99 the residual signals of DMSO- d_6 (2.49 ppm for ^1H and 39.5 ppm for ^{13}C spectra). ^{13}C NMR
100 spectra were obtained using 5K scans (acquisition time 4 h 30 min each). For ^1H - ^{13}C HSQC
101 experiments, spectra were obtained using 128 scans (acquisition time 18 h 30 min each) obtained
102 using the standard Bruker pulse program (hsqcegtpsi) with the following parameters:
103 Acquisition: TD 1024 (F2), 320 (F1); SW 10.0 ppm (F2), 160 ppm (F1); O1 2500.65 Hz; O2
104 11,318.20 Hz; D1 = 1.50 s; CNST2 = 145. Acquisition time: F2 channel, 102.55 ms, F1 channel

105 7.9511 ms. Processing: SI =1024 (F2, F1), WDW = QSINE, LB = 1.00 Hz (F2), 0.30 Hz (F1);
106 PH_mod = pk; Baseline correction ABSG =5 (F2, F1), BCFW = 1.00 ppm, BC_mod = quad
107 (F2), no (F1); Linear prediction = no (F2), LPfr (F1). Samples sizes used for these spectra ranged
108 from 10-15 mg providing NMR sample solutions with concentrations of 20-30 mg/mL.

109 **Calculating Procyanidin/Prodelphinidin (PC/PD) and *Cis/trans*-Flavan-3-ol Ratios.** The
110 percentage of PCs in the CT sample was calculated using the equation (1):

$$111 \quad \% \text{ PC} = \text{PC-6}' / [\text{PD-2}'\text{6}'/2 + \text{PC-6}'] \times 100 \quad \text{Equation (1)}$$

112 where PC-6' is the integration of the contour for the H/C-6' cross-peak of the PC units and PD-
113 2'6' is the integration of the contour for the H/C-2',6' cross-peak of the PD units. The PD-2'-6'
114 value is divided by 2 to account for the signal arising from two sets of correlated nuclei. The
115 percentage of *cis* isomers present in the CT sample was calculated through integration of the
116 respective H/C-4 *cis*- and *trans*-flavan-3-ol cross-peak contours centered around ¹H/¹³C chemical
117 shifts of 4.5-4.8/36.0 and 4.4-4.65/37.5 ppm, respectively, and used in equation (2):

$$118 \quad \% \text{ cis-flavan-3-ols} = \text{cis-flavan-3-ols} / [\text{cis-flavan-3-ols} + \text{trans-flavan-3-ols}] \times 100 \quad \text{Equation (2)}$$

119 Integrations of cross-peaks were performed in triplicate and the values were averaged.

120 Integration of the peaks was performed using Topspin 2.1 software.

121 **RESULTS AND DISCUSSION**

122 We have recently shown that ¹H-¹³C HSQC NMR spectroscopy can be a useful tool when
123 assessing the presence of CT in forages and detection of CT left in residues after HCl-butanol
124 treatment,¹⁶ demonstrating the power of 2D NMR techniques. The current study included
125 examining the ¹H-¹³C HSQC NMR spectra of 22 purified CT samples prepared from nine
126 different plant species. Based on thiolysis, the CT samples had PC/PD ratios ranging from 0/100

127 to 99/1, *cis/trans* ratios ranging from 58/42 to 95/5, and a CT content of 44 to ~100% as
128 determined by thiolysis (Table 1). As an example, ^1H - ^{13}C HSQC NMR spectrum of CT purified
129 from *Lotus pedunculatus* (big trefoil, sample number 6, Table 1) is given in Figure 2A along
130 with cross-peak assignments. The absence of significant cross-peak NMR signals from non-CT
131 organic compounds in this spectrum also confirms a high degree of purity of this sample.

132 **Determination of PC/PD Ratios.** Quantification of signals arising from polymeric materials by
133 ^1H - ^{13}C HSQC NMR spectroscopy is often hampered by nuclei having differing *T1* and *T2*
134 relaxation times and differences in coupling constants and resonance offset effects.³³ The
135 presence of these effects results in skewing of cross-peak signal contour volumes and thus
136 typically limits the utility of these contours for quantifying structural information. Usually these
137 effects require special spectroscopic treatments, alterations in NMR acquisition parameters such
138 as changes in pulse sequences or increased relaxation delays, before reliable quantification can
139 be made.³⁴⁻³⁶

140 In the ^1H - ^{13}C HSQC NMR spectra of these samples, a combination of the nuclei *T1* and
141 *T2* relaxation and resonance offset effects can be observed for most cross-peak signals. The
142 results of these effects lead to cross-peak contours in the spectra whose volumes are not
143 proportional to the corresponding nuclei ratios. As a prime example, integration of the contours
144 for signals arising from H/C-2',5' of PC units versus those from H/C-6' of PC units would
145 normally provide a ratio of 2:1 if none of the above mentioned effects were observed (Figure
146 2B). However, the integration ratios of H/C-2',5' versus H/C-6' cross-peak contours in PC
147 containing samples from this study showed wide variability with a range from 2.37:1 to 3.86:1
148 ($n = 17$, ave. = 3.15, SD \pm 0.48). Most of the signals in the ^1H - ^{13}C HSQC NMR spectra of these
149 purified CT samples followed this trend. A comparison of integration values obtained from the

150 cross-peak contours could not be directly correlated with theoretical relative intensities of the
151 nuclei giving rise to the signal. Similarly, in an attempt to assess the mean degrees of
152 polymerization (mDP) of these samples, integration of the terminal methylene unit versus any of
153 the other CT cross-peak signals in the spectra also led to no obvious correlation with the thiolysis
154 data of this study. It is worth noting that even integrations of the C-4 methylene units of the
155 flavan-3-ol monomers catechin, epicatechin and epigallocatechin under identical conditions only
156 integrate, on average, to 72% of other signals present in the ^1H - ^{13}C HSQC NMR spectrum.

157 However, integration ratios of H/C-6' cross-peak signals from PC units and the H/C-2', 6'
158 cross-peak signal from PD units did show an extremely strong and unbiased relationship with
159 PC/PD estimates from thiolysis determinations (Figure 3A). Thus, this is the first time that ^1H -
160 ^{13}C HSQC NMR data from purified CT samples have been corroborated with data from an
161 alternative method (thiolysis) to quantify compositional characteristics of CTs. Separate NMR
162 analyses conducted on a limited set of other purified CT samples at the University of Reading
163 confirmed this method as providing reliable PC/PD ratios.

164 It is not clear how all of the parameters controlling contour intensities are interrelated: do
165 the nuclei involved impart the same or similar $T1$ and $T2$ relaxation times, coupling constants
166 and resonance offset effects, allowing for accurate comparison of the two contours, or is this
167 simply a coincidence of cancellation of the effects? Answers to these questions remain to be
168 determined.

169 To test for variability in sample to sample preparation and data acquisition, we prepared
170 duplicate NMR solutions from the same CT samples and obtained NMR spectra of these
171 preparations on different days. These results are given in Table 2. As shown, there is excellent

172 reproducibility of the method between these duplicate runs. In all, these experiments prove that
173 this is a robust method for estimation of PC/PD ratios in purified CT samples.

174 **Determination of *Cis/Trans* Flavan-3-ol Ratios.** In order to assess *cis*- and *trans*-flavan-3-ol
175 ratios (i.e. ratio of epicatechin and epigallocatechin versus catechin and galocatechin) in these
176 samples we focused on the H/C-4 cross-peak signal (Figure 2C). It has been reported³² that this
177 signal is segregated into two cross-peaks with ¹H/¹³C chemical shifts of ~4.5-4.8/36.0 and ~4.4-
178 4.65/37.5 ppm for the *cis*- and *trans*-flavan-3-ol subunits, respectively. The integration of cross-
179 peak signals in ¹H-¹³C HSQC NMR spectra of the same nuclei with the same connectivity in near
180 identical electronic environments should be straight-forward as they should possess similar, if
181 not identical, *T1* and *T2* relaxation times and pose little or no differences in coupling constants
182 and resonance offset effects. Thus, we should be able to use the data obtained from these ¹H-¹³C
183 HSQC NMR spectra to directly measure this structural element of isolated CTs. The percentage
184 of *cis* isomers present in the CT sample was calculated through integration of the respective H/C-
185 4 *cis* and *trans* cross-peak contours (Figure 2C). Integration ratios from these contours provided
186 strongly related but biased estimates of *cis/trans* ratios relative to thiolysis (Figure 3B). A
187 literature search revealed that this segregation of the *cis* and *trans* signals of flavan-3-ol moieties
188 is most likely not absolute and this could provide an explanation for the bias in *cis/trans*
189 estimates relative to thiolysis. NMR spectroscopic data from epicatechin (*cis*) oligomers report
190 ¹³C chemical shift in the range of 37.5 ppm, overlapping into the previously designated “*trans*”
191 signal region.^{37,38} The lack of signal segregation is more pronounced in structures containing C4-
192 C6 interflavanyl linkages.^{38,39} Thus, overlapping of signals from *cis*- and *trans*-flavan-3-ol
193 subunits is the most likely contributing factor for slightly larger discrepancies between the

194 thiolysis/NMR correlations for *cis/trans*-flavan-3-ol subunit assessments, and may also be
195 responsible for the biased regression fit (Figure 3B).

196 **Precautions.** The first issue here, as with most analytical techniques, is to obtain a spectrum with
197 strong signal to noise ratio before attempting to integrate the data. If sample size is limited,
198 extended acquisition times need to be considered. When using this technique on samples of low
199 purity it is imperative that the user be able to recognize any non-CT impurity signals present and
200 avoid incorporating them into the integration values. For PC/PD ratio evaluations, we have found
201 that the signals indicated in Figure 2B are the most common impurity signals which may
202 interfere in obtaining reliable results. These signals most likely arise from trace amounts of non-
203 CT polyphenols present in the sample. For the assessment of *cis/trans* ratios, the problem of
204 integration of non-CT impurities does not seem to be an issue. The H/C-4 cross-peak signals
205 appear, even in spectra of whole plant material, in an area void of other non-CT signals. The
206 major issue in the *cis/trans* ratio assessment is the resolution of the two signals. In some cases
207 these signals are not well resolved (Figure 3B) and care needs to be taken in selecting the
208 integration areas.

209 In conclusion, the method developed now permits analytical assessment, via 2D ^1H - ^{13}C
210 HSQC NMR spectroscopy, of two specific chemical properties of purified CT samples: PC/PD
211 and *cis/trans* ratios. Purified CT samples examined encompass the entire range of
212 procyanidin/prodelphinidin ratios from 0/100 to 99/1 and a substantial range of *cis/trans*-flavan-
213 3-ol ratios from 58:42 to 95.5:4.5. The observations outlined here also provide validation of
214 thiolysis data for analysis of CT composition. In contrast to thiolysis, NMR spectroscopy
215 represents a non-destructive analytical tool, which can be important when sample quantities are
216 limited. Thiolysis requires ca 4 mg for a single determination, whereas NMR analysis requires

217 only 10 mg for an 18 h acquisition time using the described instrumentation. No additional
218 straight-forward correlations were found upon examination of other cross-peak signals in these
219 ^1H - ^{13}C HSQC NMR spectra. Additional spectroscopic examination of these samples is
220 warranted to investigate whether other significant structural information can be obtained using
221 quantitative ^1H - ^{13}C HSQC NMR data³⁴⁻³⁶ or alternative NMR techniques.

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234 Mention of trade names or commercial products in this article is solely for the purpose of
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236 Department of Agriculture.

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ABBREVIATIONS USED

241 ^1H - ^{13}C HSQC, proton-carbon-13 heteronuclear single quantum coherence ; NMR, nuclear
242 magnetic resonance; PC, procyanidin; PD, prodelphinidin; *cis*, 2,3-*cis*; *trans*, 2,3-*trans*; CT,
243 condensed tannins; mDP, mean degree of polymerization; ^{13}C , carbon-13; CPMAS, cross
244 polarization magic angle spinning; 1D, one dimensional; 2D, two dimensional; 5K, five
245 thousand; DMSO-*d*₆, perdeuterated dimethyl sulfoxide.

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Figure 1. Structures of common flavan-3-ol monomeric subunits found in condensed tannins (left). A condensed tannin tetramer (right) showing C4-C8 (B-Type) linkages, PC and PD extender units and a terminal unit.

Figure 2. (Panel A) Signal assignments for the ^1H - ^{13}C HSQC NMR spectrum (500/125 MHz, DMSO- d_6 /pyridine- d_5 , 4:1) of purified condensed tannin sample (Table 1, Sample Number 6) from *Lotus pedunculatus* (big trefoil) leaves; (Panel B) B-Ring aromatic region cross-peak signals including H/C-2',6' PD signal and the H/C-2',5' and 6' signals from procyanidin units; and (Panel C) H/C-4 *cis*- and *trans*-flavan-3-ol cross-peak signals. Contours were integrated as indicated by boxes. Non-tannin related signals arising from impurities are noted and are not included in the integration.

Figure 3. (Left Panel) Proportion of procyanidin subunits in 22 isolated condensed tannin samples as determined by thiolysis vs. ^1H - ^{13}C HSQC NMR. (Right Panel) Proportion of *cis* subunits in 22 isolated condensed tannin samples as determined by thiolysis vs. ^1H - ^{13}C HSQC NMR.

Table 1. Comparison of Data from Thiolytic and ¹H-¹³C HSQC NMR Determinations for 22 Condensed Tannin (CT) Samples.

CT Sample Number	CT content (thiolysis) (%)*	SD	% PC (thiolysis)		% PC (NMR)		% cis (thiolysis)		% cis (NMR)		
			plant species	SD	SD	SD	SD	SD	SD		
1	<i>Lespedeza cuneata</i>	96.3	0.08	5.9	0.06	4.9	0.06	79.2	0.26	73.2	1.19
2	<i>Lotus corniculatus</i>	92.5	0.03	54.0	0.62	55.6	1.17	93.3	0.58	86.8	0.59
3	<i>Lotus corniculatus</i>	78.1	0.40	68.0	0.35	70.9	0.19	87.5	0.15	88.7	1.17
4	<i>Lotus corniculatus</i>	75.3	0.01	57.1	0.12	60.8	0.32	91.3	0.13	87.0	1.58
5	<i>Lotus pedunculatus</i>	108.0	0.01	16.0	0.07	14.6	0.80	81.7	0.22	69.3	1.63
6	<i>Lotus pedunculatus</i>	91.3	0.35	25.9	0.29	23.7	0.28	78.7	0.23	75.4	1.22
7	<i>Lotus pedunculatus</i>	85.8	0.01	17.5	0.06	17.5	0.50	79.5	0.05	71.7	0.45
8	<i>Lotus pedunculatus</i>	80.3	0.41	28.1	0.17	29.0	1.02	74.4	0.15	71.0	1.79
9	<i>Onobrychis viciifolia</i>	102.2	8.13	37.3	0.29	39.1	3.23	82.9	0.27	84.4	5.05
10	<i>Onobrychis viciifolia</i>	93.7	4.55	19.2	0.06	19.1	0.28	83.3	0.21	77.9	1.96
11	<i>Onobrychis viciifolia</i>	82.4	1.10	51.7	0.32	56.7	0.62	83.5	0.10	79.7	0.90
12	<i>Onobrychis viciifolia</i>	44.3	0.17	57.3	0.07	59.0	1.45	68.7	0.00	64.9	1.10
13	<i>Securigera varia</i>	56.6	n=1	18.2	n=1	22.5	0.17	89.7	n=1	87.6	0.56
14	<i>Sorghum bicolor</i>	58.8	0.02	100.0	0.00	100.0	0.00	85.5	0.09	87.1	2.60
15	<i>Theobroma cacao</i>	63.8	n = 1	100.0	n=1	100.0	N	93.4	n = 1	100.0	N
16	<i>Theobroma cacao</i>	49.0	0.01	100.0	0.0	100.0	0.00	90.1	0.12	88.7	2.06
17	<i>Tilia sp.</i>	92.7	0.04	98.5	0.05	99.2	0.19	95.5	0.09	91.2	0.15
18	<i>Tilia sp.</i>	61.1	0.47	98.1	0.14	99.2	0.47	89.4	0.11	89.1	0.73
19	<i>Trifolium repens</i>	120.6	0.01	0.8	0.00	0.0	N	69.3	0.07	61.1	1.01
20	<i>Trifolium repens</i>	111.4	4.80	1.3	0.00	0.0	N	58.9	1.27	56.3	0.75
21	<i>Trifolium repens</i>	106.6	5.08	0.9	0.04	0.0	N	58.3	0.24	50.6	1.22
22	<i>Trifolium repens</i>	97.6	0.01	1.1	0.04	0.0	N	69.8	0.02	56.1	1.60

N = Not detected; ND = not determined as based on single analyses Note: % purity refers to g tannins/100 g fraction; % PD = 100 -

% PC; % trans = 100 - % cis.

Table 2. Comparison of Duplicate NMR Data with Thiolysis Data Obtained from Condensed Tannin (CT) Samples (% PC = Percentage of Procyanidins in CT Sample; % *cis* = Percentage of *cis*-flavan-3-ols in CT Sample).

CT Sample	% PC		% PC		% <i>cis</i>		% <i>cis</i>	
Number	(thiolysis)	SD	(NMR)	SD	(thiolysis)	SD	(NMR)	SD
3	68.0	0.35	70.0	0.49	87.5	0.15	88.6	1.32
3			71.1	0.50			88.6	0.68
4	57.1	0.12	60.4	0.41	91.3	0.13	89.8	0.53
4			59.7	0.53			91.1	0.50
6	26.0	0.29	24.4	0.35	78.7	0.23	75.4	1.02
6			23.7	0.17			75.1	0.99
7	17.5	0.06	17.5	0.50	79.5	0.05	71.6	0.45
7			18.6	0.47			71.8	2.20
11	51.7	0.32	56.9	0.42	83.5	0.10	79.5	1.20
11			55.5	0.13			80.9	0.68

Note: Percentages for prodelfinidins (PD) and *trans* flavanols are not shown as % PD = 100 - % PC and % *trans* = 100 - % *cis*.

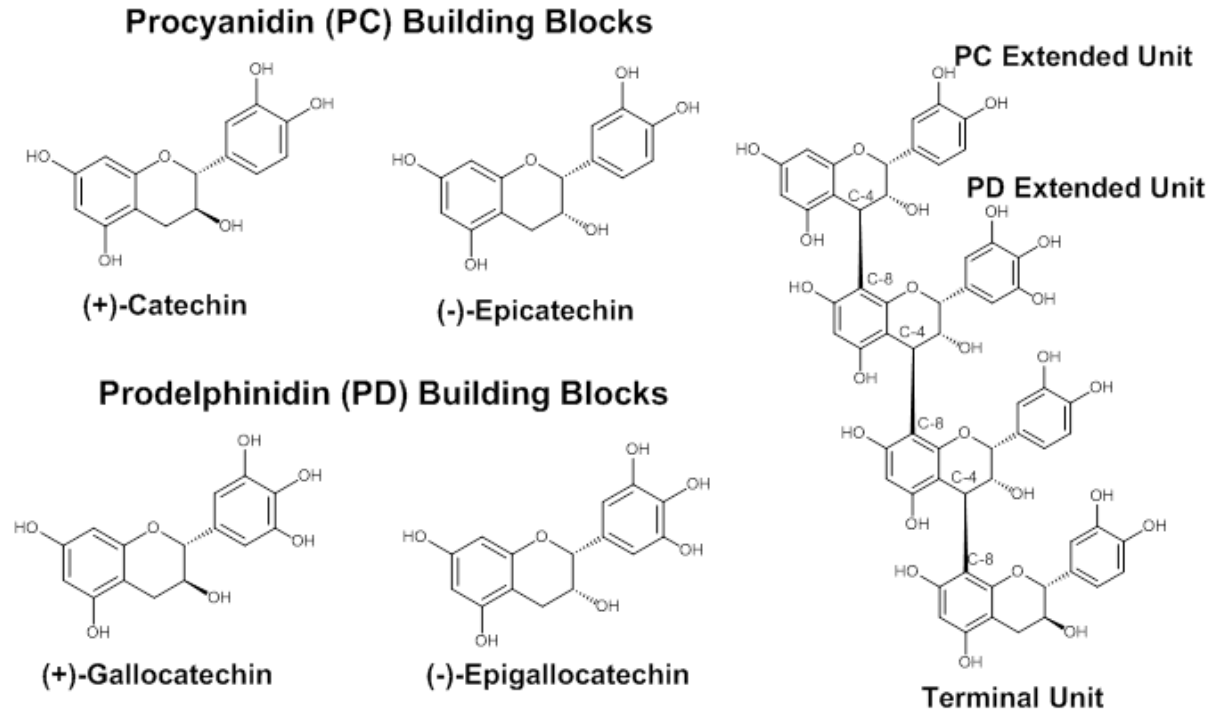


Figure 1.

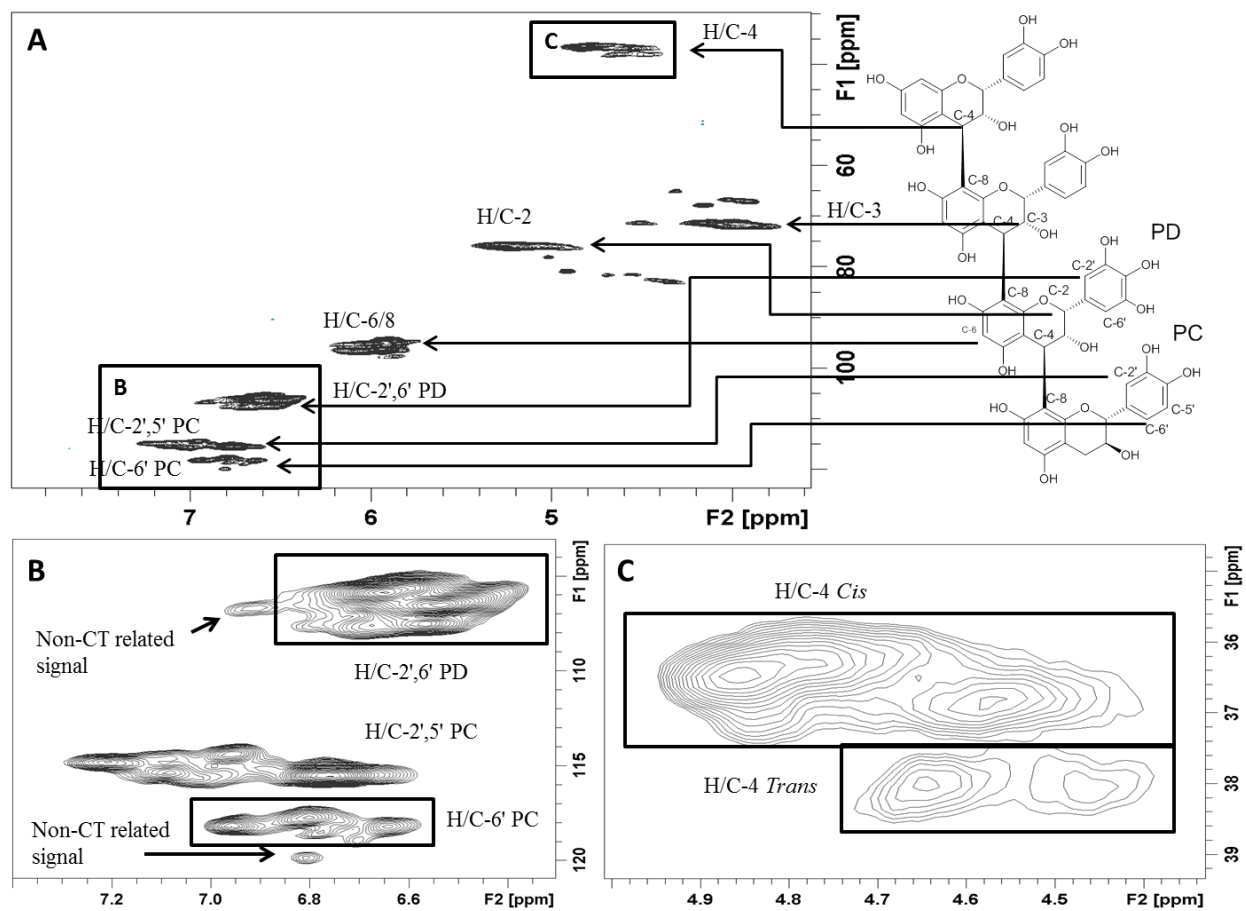


Figure 2.

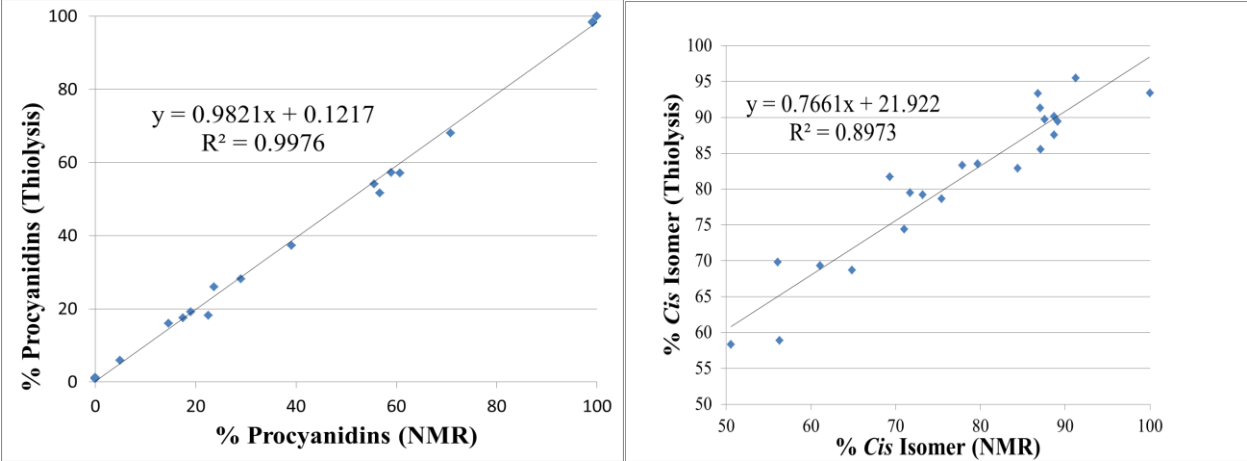


Figure 3.

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