Carbon-13 cross-polarization magic-angle spinning nuclear magnetic resonance (CPMAS NMR) for measuring proanthocyanidin content and procyanidin to prodelphinidin ratio in sainfoin (Onobrychis viciifolia) tissues

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Carbon-13 Cross-Polarization Magic-Angle Spinning Nuclear Magnetic Resonance (CPMAS NMR) for Measuring Proanthocyanidin Content and Procyanidin to Prodelphinidin Ratio in Sainfoin (*Onobrychis viciifolia*) Tissues

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ABSTRACT

A procedure based on ^{13}C CPMAS NMR was developed to study procyanidins (PCs) and prodelphinidins (PDs) directly in milled sainfoin plant tissues. Blackcurrant and *Tilia* samples enabled reference spectra of purified proanthocyanidin (PA) fractions, crude extracts and milled plant tissues, with characteristic resonances at 155, 144 and 132 ppm. PC/PD ratios were estimated from the I_{132}/I_{155} intensity ratio and differed by 2.5 to 5.9% compared to thiolysis data. Normalization to the 155 ppm signal intensity from reference spectra enabled analysis of PA contents with an error of ca 8 g PAs/100 g plant tissue. The procedure estimates the lignin contribution and allows for a correction of the PA content. In six sainfoin accessions, estimated PA contents, were 1.6- to 20.8-fold higher than the thiolysis and 1.4- to 2.6-fold higher than the HCl-butanol-acetone results. Method differences may reflect the presence of unextractable, possibly high molecular weight PAs in sainfoin.

KEYWORDS: *condensed tannins; CPMAS NMR; thiolysis; HCl-butanol-acetone assay; extractable and non-extractable proanthocyanidins; sainfoin;*

INTRODUCTION

Given the beneficial effects of proanthocyanidins (PAs) on human and animal nutrition and health,[1, 2] there is a need for analytical methods capable of providing information on the true PA content and their composition in plants. However, their analysis is challenging as many plants synthesize complex polymeric mixtures of PAs (Figure 1).[3, 4] Extractable PAs are typically analyzed after degradation in the presence of nucleophiles followed by chromatographic separation of the reaction products and mass spectrometric analysis; this gives data on PA content, flavan-3-ol subunit composition, mean degree of polymerization (mDP) and procyanidin/prodelphinidin (PC/PD) and *cis/trans* flavan-3-ol ratios.[5-8] Extraction, however, is often incomplete as many plants also contain large quantities of unextractable PAs, which can lead to underestimation of total contents.[9-11] Therefore, *in planta* PA depolymerization with HCl-butanol-acetone, thiolysis or phloroglucinolysis has been used to estimate extractable plus unextractable PAs; but, incomplete reaction, side reactions or degradation-resistant PAs continue to pose challenges.[3, 4, 12-14] Of particular concern is the fact that the HCl-butanol-acetone reagent often, but not always, gives higher PA contents than thiolytic degradation;[15, 16] although opposite results have also been found with galloylated PAs (unpublished results). Questions remain, therefore, about the true PA contents in plants and foods. Currently, little is known about the quantities and composition of unextractable PAs and alternative methods deserve evaluation.[10, 17]

In contrast to the above degradation methods, nuclear magnetic resonance (NMR) has the potential to provide direct evidence and accurate results for total, i.e. extractable plus unextractable, PAs. Solution-state ^{13}C NMR spectroscopy has been used to determine the relative purities,[18, 19] PC/PD[18-21] and *cis/trans* flavan-3-ol ratios[18, 20, 21] and mDP values of extracted PAs.[20, 21] Whilst two dimensional gel-state NMR proved useful for assessing the presence and composition of PAs in *Lotus* tissues,[22] our proof-of-concept study showed that

gel NMR spectra of sainfoin (*Onobrychis viciifolia*) tissues had signal resolution and matrix interference problems.

Solid-state NMR experiments have been used for the direct analysis of plant materials and require neither extraction nor derivatization of PAs.[23-25] Cross-polarization magic-angle spinning (CPMAS) NMR techniques have previously been used to evaluate the efficiency of PA extraction in pine barks,[23] softwood and barks from quebracho[25] and humus[26] as well as to characterize PAs in dietary fiber samples.[27] In addition, differences in fingerprint spectra were used to distinguish between PA transformations of leather tannings[28] and during fungal degradation.[29] However, significant problems with poor resolution and frequent overlaps of multiple signals prevented any in depth quantitative estimates of the PA content and PC/PD ratios.

Sainfoin is a PA-containing forage legume, which is of interest for its nutraceutical and environmental properties. It engenders high voluntary intakes,[30, 31] has excellent nutritive value,[30, 31] lowers *in vitro* greenhouse gas emissions,[32] prevents bloat in ruminants,[33] lowers gastrointestinal nematodes,[34] and can enhance the unsaturated fatty acid composition of meat and milk products.[35, 36] The work presented here used six sainfoin accessions as a case study for complex PA mixtures.[37, 38] Such complexity may have been the main reason for the observed discrepancies between the thiolysis and HCl-butanol-acetone assay results and still requires detailed experimental confirmation.[39] Therefore, the aim of this study was to develop a procedure based on a ^{13}C CPMAS NMR technique for estimating PA content and composition directly, without chemical modification in different sainfoin accessions and to compare the results to thiolysis and HCl-butanol-acetone data.

MATERIALS AND METHODS

Plant Samples. Blackcurrant (*Ribes nigrum*) leaves were collected from Hildred PYO farm (Goring-on-Thames, UK). *Tilia* (*Tilia* spp.) flowers were purchased from Flos (Mokrsko, Poland) as described by Ropiak et al.[8] Sainfoin (*Onobrychis viciifolia*) leaves and stems, from six accessions, were provided by the National Institute of Agricultural Botany (Cambridge, UK). Blackcurrant leaves and sainfoin plants were lyophilized or air-dried and subsequently ground to pass a 1 mm sieve and ball-milled. Fine powders of ball-milled blackcurrant leaves and *Tilia* flowers were also mixed on a weight basis (100/0, 70/30, 50/50, 30/70 and 0/100) for NMR analysis and the development of the procedure to elucidate PC/PD ratios.

Chemicals and Reagents. Hydrochloric acid (36%), formic acid, acetic acid, butan-1-ol, HPLC-grade acetone, HPLC-grade methanol, HPLC-grade dichloromethane, HPLC-grade hexane, HPLC-grade acetonitrile and ammonium chloride were purchased from Fisher Scientific Ltd (Loughborough, UK). (±) – Dihydroquercetin (98%) was from Apin Chemicals (Abingdon, UK). Benzyl mercaptan and acetone-d_6 (99.9%) were supplied by Sigma-Aldrich (Poole, UK). Deuterium oxide (D_2O) was from CK Isotopes (Ibstock, UK). Sephadex LH-20 was purchased from GE Healthcare (Little Chalfont, UK). Deionized water was purified in a Milli-Q system (Millipore, Watford, UK).

Proanthocyanidin Extraction and Purification. Finely ground plant tissue (50 g) was weighed into a conical flask. Acetone/water (500 mL, 7:3, v/v) was added and the mixture was vigorously stirred for 1 h. The mixture was filtered under vacuum. The filtrate was further extracted with dichloromethane (250 mL) to remove lipids and chlorophyll. Polyphenols were concentrated in the aqueous phase with a rotary evaporator below 37.5 °C to yield the "crude extract".

This crude extract was diluted with deionized water (2 L) and filtered under vacuum. The filtrate was loaded on a 400 x 65 mm i.d., glass column which had been packed with 70 x 65 mm i.d., Sephadex LH-20 resin and was equipped with a sintered-glass frit.[40] The column was then washed with deionized water (2 L) to elute sugars and low molecular mass phenolics.[40] Gravity elution with acetone/water (3:7, 1:1 and 8:2, v/v; 1 L each) yielded three PA fractions from each plant source; the first 200 mL of each eluate was discarded and PA fractions 1, 2 and 3 were collected in the next 500, 300, and 300 mL, respectively. The organic solvent was removed on a rotary evaporator below 37.5 °C and the PA fractions were frozen, freeze-dried and stored at -20 °C. Fractions 2 that eluted with 1:1 acetone/water were designated as "purified PA fractions". Various aliquots from the purified blackcurrant and *Tilia* PA fractions (152:0, 36:83, 45:38, 63:25, 0:157 mg) were also mixed, in order to provide a range of nominal PC/PD values of 0/100, 30/70, 50/50, 70/30 and 100/0, for NMR analysis. The calculated PC and PD contents within purified PA fraction (in g PCs or PDs/100 g purified PA fraction) were based on the PC and PD contents from thiolysis (*Tilia*: 97.4 g PCs/100 g of purified PA fraction and blackcurrant: 94.9 g PDs/100 g of purified PA fraction).

Thiolysis of Purified Proanthocyanidins. Thiolysis reactions on purified PA fractions that eluted with acetone/water (1:1 v/v) were performed according to Novobilský et al.[41] Purified PA fractions (4 mg) were weighed into a 10 mL screw-capped vial, dissolved in methanol (1.5 mL) and acidified with HCl (0.5 mL, 3.3% in methanol, v/v). This was followed by addition of benzyl mercaptan (50 μL) and the reaction mixture was stirred in a water-bath for 1 h at 40 °C. The reaction was quenched by adding ultrapure water (2.5 mL) to the mixture at room temperature. The analysis and quantification of thiolysis reaction products were

144 performed with reverse-phase HPLC according to Gea et al.[5] The operating conditions and
145 parameters of HPLC analysis were described by Williams et al.[42]
146
147 ***In planta* Thiolysis of Sainfoin Proanthocyanidins.** Milled freeze-dried sainfoin plant
148 material (200 mg) was weighed into a 10 mL screw-capped vial, dissolved in methanol (2
149 mL) and acidified with HCl (1 mL, 3.3% in methanol, v/v). This was followed by addition of
150 benzyl mercaptan (100 µL) and the mixture was stirred in a water-bath for 1 h at 40 °C. The
151 reaction was stopped by transferring the vials into an ice-bath and 1% formic acid in
152 ultrapure water (9 mL) was added at room temperature. The samples were centrifuged (3000
153 rpm, 5 min) and 1 mL of the mixtures added to HPLC vials for LC-MS analysis.
154 Samples (5 µL) were injected into an 1100 series HPLC system (Agilent
155 Technologies, Stockport, UK) connected to a 150 x 3 mm i.d., 5 µm, ACE super C_{18} column
156 (Hichrom, Theale, UK) fitted to a 150 x 3 mm i.d., 5 µm, ACE EXCEL Ultra-Inert UHPLC
157 compatible guard cartridge (Hichrom, Theale, UK). The column temperature was set at 60
158 °C. The HPLC system consisted of a G1379A degasser, a G1312A binary pump, a G1313A
159 ALS autoinjector, a G1314A VWD UV detector and a G1316A column oven and an 1100
160 series MSD API-ES mass spectrometer (Agilent Technologies, Waldbronn, Germany). Data
161 were acquired and processed with ChemStation software (version A 10.01 Rev. B.01.03).
162 The flow rate was 0.4 mL/min using formic acid (1%) in water containing 100 mg/L
163 ammonium chloride (solvent A) and HPLC-grade acetonitrile (solvent B). The thiolysis
164 reaction products eluted with the following gradient: 0-7 min, 2.5% B; 7-15 min, 2.5-5% B;
165 15-22 min, 5-10% B; 22-40 min, 10-40% B; 40-45 min, 40-100% B; 45-49, 100-2.5% B; 49-
166 60, 2.5% B. Mass spectra were recorded in the negative ionization scan mode from *m/z* 100-
167 1000. The mass spectrometer operating conditions were as follows: 3000 V for capillary
168 voltage, nebulizer gas pressure at 35 psi, drying gas at 12 mL/min and dry heater temperature

at 350 °C. Flavan-3-ols and their benzyl mercaptan adducts were identified by their retention times, ultraviolet-visible (UV) spectra recorded at 280 nm and molecular masses and they were quantitated using published response factors against dihydroquercetin.[5, 8]

***In planta* Sainfoin Proanthocyanidin Analysis with the HCl-Butanol-Acetone Assay.** The HCl-butanol-acetone assay followed the procedure of Grabber et al.[22] with minor modifications. Briefly, lyophilized sainfoin plant tissue was weighed (10 mg) into a 10 mL screw-capped vial. The reagent mixture was prepared by mixing ammonium ferric sulfate (150 mg) in ultrapure water (3.3 mL), hydrochloric acid (12 M, 5 mL), butan-1-ol (42 mL) and acetone (50 mL). An aliquot of the reagent (10 mL) was added to the sainfoin sample. The sample was left at room temperature (1 h) to check for the presence of flavan-4-ols or flavan-3,4-diols as these generate false positives.[43] Tubes were then stirred and heated at 70 °C for 2.5 h in the dark. Samples were left to cool to room temperature and centrifuged (3000 rpm, 1 min). Absorbance of the supernatant was recorded by scanning from 450-650 nm using a V530 spectrophotometer (Jasco, Great Dunmow, UK). The HCl-butanol-acetone reagent was used as a blank and all samples were run in duplicate. A sainfoin sample of known PA content and composition was used for quality control purposes.

Analysis of Proanthocyanidins by ^{13}C Cross-Polarization Magic-Angle Spinning Nuclear Magnetic Resonance. The solid-state CPMAS NMR spectra were recorded on a Bruker Avance III 500 MHz spectrometer (Bruker Biopsin, Rheinstetten, Germany) operating at carbon-13 Larmor frequency of 125.78 MHz (11.75T). Crude extracts and purified PA fractions from *Tilia* and blackcurrant and ball-milled *Tilia*, blackcurrant and sainfoin plant tissue samples (~80 mg) were compressed into standard 4 mm zirconia rotors prior to analysis. A standard bore Bruker MAS probe was used and rotors were spun at 10 kHz rate.

194 The proton 90° pulse width was 3.7 μs at a power level of 32 W. The variable amplitude CP
195 ramp (90-100) was used with a contact time of 1 ms. In total, 4096 signal transients were
196 averaged into each spectrum with a 6 s relaxation delay at ambient temperature. All spectra
197 were referenced to an external adamantane signal (at 38.5 ppm with respect to TMS) as a
198 secondary reference. Dipolar dephasing experiments were run with identical parameters to
199 ^{13}C CPMAS NMR experiments and dephasing filter time (tdd) of 45 μs was optimized to
200 attenuate the signals of all protonated carbons.

201 To accurately extract intensity of all signals, each experimental spectrum was fitted
202 with a calculated spectrum that was constructed from the sum of signals with a Gaussian line
203 shape. Numbers and positions of signals in the calculated spectrum corresponded directly to
204 the number and positions of signals in the experimental spectrum. The width, amplitude and
205 position of each signal in the calculated spectrum was optimized by minimizing the
206 discrepancy between the experimental and calculated spectra using an "in house" written
207 Matlab routine based on the least square method. A number of initial parameters were tested
208 and the sets with the smallest discrepancy led to final results with a narrow range of the
209 fitting error. Once the width and the amplitude of the fitted signal at 155 ppm were known,
210 the intensity was calculated.

211 The PA content of sainfoin plant tissue samples was estimated by comparing the
212 signal intensity of the reference signal I_R at 155 ppm (from *Tilia* with 95 g PAs/100 g of
213 purified PA fraction, determined by thiolysis) with the intensity I_S of the 155 ppm signal in
214 the investigated sample.

215 The calculation of experimental error involved three factors: (i) the non-strictly
216 quantitative character of the solid-state NMR experiments because of the cross-polarization
217 signal enhancement (and possibly the miscalibration of the relaxation parameters); (ii) the

small variation in setting hardware parameters, such as tuning and matching of the probe; and (iii) the numerical fitting of the spectra.

The recording of 2-3 spectra of the same sample with slightly different settings regarding the first two factors (including contact time and relaxation delay) resulted in an error of ca 2%. The discrepancies of the spectra acquired with different parameters introduced an additional error of ca 2% after the fitting process. This accounted for a total error of ca 4% for the intensity measurements. The PA content was calculated by comparing the reference signal intensity to the intensity of the sample of interest. Therefore, the total experimental error (ca 8% or 8 g PAs/100 g of plant tissue) derived from the sum of the intensity errors between reference and sample intensity.

The influence of the spinning sidebands on the signal intensity was assessed by comparing the 155 ppm signal intensity recorded at 10, 12 and 15 kHz spinning rates for both crude extract and sainfoin plant tissue samples. Only small differences of less than 1%, well within the experimental error were observed. At 10 kHz spinning rate, the spinning sidebands of the signal at 155 ppm were not fully removed and decreased the intensity by ca 12%. However, the identical anisotropy of the chemical shift of C5, C7 and C8a carbons in purified PA fraction and plant tissue PAs is expected (due to the same molecular structure) and by normalizing the intensity of the 155 ppm signal from the sample of interest to the high purity reference with 95 g PAs/100 g of purified PA fraction from *Tilia*, the contribution of these factors to the intensity error could be neglected.

However, it should be noted that ^{13}C CPMAS NMR is not strictly quantitative and, therefore, it is unlikely but not impossible that the discrepancies could have exceeded to a small extent the range of the estimated experimental error.

RESULTS AND DISCUSSION

Assignment of Signals in the Solid-State NMR Spectrum. The ^{13}C CPMAS NMR spectra of the purified PA fractions from *Tilia* and blackcurrant consisted of approximately ten resolved resonances each centered between 170 and 0 ppm and showed several PA fingerprint signals (Figure 2B). Characteristic resonances at 155, 144 and 132 ppm were clearly observed in the spectra of all three sample types, i.e. purified PA fractions, crude extracts and plant tissues (Figure 3). The signal at 155 ppm originated from the non-protonated carbons C5, C7, C8a of the flavan-3-ol structure (Figure 2A).[25, 27, 44] The signal at 144 ppm was from the resonance of the non-protonated carbons C3′, C5′ of blackcurrant PAs and the non-protonated carbons C3′, C4′ of *Tilia* PAs.[24, 25, 44] The most distinctive signal at 132 ppm originated from the non-protonated carbons C1′, C4′ in blackcurrant PAs but only from the non-protonated carbon C1′ in *Tilia* PAs.[24, 25, 44]

In order to confirm these assignments, dipolar dephasing solid-state NMR experiments were performed. The experimental parameters were optimized to detect only signals from non-protonated carbons (Figure 2B). The resulting data confirmed our initial identification and were in good agreement with spectra in reports on pecan nutshell PDs[45] and *Photinia* leaf PCs.[44] Weak signals of not completely removed spinning sidebands were detected in the 80-40 ppm region. Some very weak contributions from the protonated carbons were also visible in the 40-20 ppm region and could have been caused by a small miscalibration of parameters for the dephasing filter.

The resonances from protonated carbons were spread across the 120-0 ppm region and showed line broadening and overlap, especially for the crude extracts and milled plant tissues (Figures 2B and 3). This was likely due to interferences from other plant components such as lignin, pectin, cutin, cellulose and hemicellulose as previously reported in crude extracts and milled plant samples.[25, 28, 44] However, our signal assignments of the purified PA

fractions from *Tilia* and blackcurrant were consistent with the much better resolved ^{1}H-^{13}C HSQC solution NMR spectra of the same samples.

Estimation of Proanthocyanidin Content in the Presence of Lignin. Accurate quantification of PAs in plant matrices is of great interest and several techniques have been used including solid-state NMR. The region between 160-120 ppm is understood to be the most suitable despite some possible lignin or lignin-PA complex contributions especially in milled plant samples.[25, 44] In particular, Wawer et al.[27] postulated that resonances at 155 and 144 ppm in solid-state NMR dipolar dephased spectra from *Aronia* and blackcurrant fiber powders could include responses from C3, 5-OMe and C4-OH carbons of lignin. However, this interference was on a smaller scale (i.e. there were no significant changes to the signal pattern) compared to resonances below 120 ppm (Figure 3) where the appearance changed completely between purified PA fractions and milled plant tissues.

The characteristic signals at 155, 144 and 132 ppm in the spectra of the purified PA fractions could only have derived from PAs as large polymeric lignin molecules were not expected to be soluble in acetone/water.[27, 46] Our strict, two step purification process also assured that the low molecular weight phenolics or lignin fragments which may have been present in small quantities in crude extracts were predominantly removed in the purified PA fractions. The intensities of the 132 ppm signal spinning side bands at 211 ppm (left spinning side band) and 53 ppm (right spinning side band) were exactly the same (0.02% difference) which proves that there is no, or only negligible, lignin contribution to the NMR spectra of the purified PA fractions. Figure 3 shows the comparison of the normalized amplitude of these characteristic signals between purified PA fractions, crude extracts and milled plant tissues. However, it was not possible to estimate the contribution of impurities or plant matrix components to the intensity because currently there is no technique that can provide

completely accurate data on PA concentrations in the plant tissues. However, a contribution of lignin in this region of the spectrum was expected between 155 and 144 ppm as postulated,[27] and it should affect the width of the resonances due to overlap. Therefore, measuring the change of the signal width in purified PA fractions, crude extracts and milled plant tissues could provide an estimate of lignin interference. In our case, the estimated increase in the width of the 155 and 144 ppm signals was <15% for crude extracts and <25% for plant tissues, and corresponded well to the increased intensity of the lignin signal at 53 ppm, which was especially evident for the *Tilia* plant tissues (Figure 3B).

This simple estimate showed that the lignin interference had to be taken into account as a correction to the PA content in plant samples. To test the accuracy of this assumption, the content of extractable PAs in the crude extract from blackcurrant was estimated with ^{13}C CPMAS NMR and compared to the thiolysis result. The PA content determined by NMR without correction was 39.7 g PAs/100 g crude extract and with the 15% reduction was 33.7 g PAs/100 g crude extract (with an error of ± 8 g PAs/100 g). The latter value was in excellent agreement with the thiolysis result of 29.2 g PAs/100 g crude extract. This demonstrates that both techniques can be used for the analysis of extractable PAs.

Although this simple calculation may take into account a lignin contribution, other factors could also influence the width of the resonance line, e.g. relaxation and mobility issues can arise from large polymeric molecules[25] and small variation in the shimming quality.[47] Further, one needs to be aware that the thiolysis and HCl-butanol-acetone assays have their own limitations, especially when applied directly to plant tissues.

Despite problems arising from possible plant matrix interferences in the milled plant tissues, it may be concluded that the 155 ppm signal of the purified PA fractions was a suitable marker to estimate PA contents in unknown milled plant tissues. Therefore, the PA content of sainfoin plant tissue samples was estimated by comparing the intensity of a

317 reference signal, I_R, of the purified PA fraction (95 g PAs/100 g as determined by thiolysis)
318 from *Tilia* and the intensity of the I_S signal of the investigated sample.[23] The content error
319 was estimated to be ca 8 g PAs/100 g plant tissue.

320 The PA content of selected sainfoin plant tissues determined by ^{13}C CPMAS NMR
321 (without and with a 25% reduction for the lignin correction) was then compared to results
322 from the thiolysis and the HCl-butanol-acetone assays (Table 1). Selected spectra are shown
323 in Figure 4. Clearly, solid-state NMR consistently gave the largest PA content in all sainfoin
324 plant tissue samples and thiolysis gave the smallest. Most HCl-butanol-acetone values were
325 higher than the thiolysis data[16] and accounted for ca 50% of the NMR data after lignin
326 correction. The results varied greatly between the sainfoin accessions for all methods, but it is
327 worth pointing out that the three Zeus samples (S1, S2 and S6) gave consistent results with
328 the NMR and HCl-butanol-acetone but not the thiolysis technique.

329 The above results illustrate the complexity of determining PA content directly in
330 plants. Our NMR results can be regarded as an upper limit of PA content compared to the
331 other techniques (Table 1) and is more likely to measure previously undetected unextractable
332 PAs because NMR does not require any chemical modification of the sample. Solid-state
333 NMR has already proved useful for estimating differences in PA contents within species, i.e.
334 for samples with similar PA compositions.[25, 28] Our approach to account for a lignin
335 contribution could improve the way NMR could be used for PA measurements more
336 generally, including for the first time full quantitative elucidation. The validity of our
337 approach is illustrated by the analysis of the amplitude of the lignin signal at 53 ppm for
338 samples S1, S3 and S4 (Figure 4). The strongest lignin signal was detected in the S1 sample
339 and the weakest and approximately equal signals in the S3 and S4 samples. Had lignin
340 interference been the predominant factor in the measurement of PA content then S1 should
341 have had more PAs than the S3 and S4 samples. As evident from the 155 and 145 ppm

signals this was not the case as the S3 sample with the smallest lignin contribution had the largest PA content.

There are several possible reasons for the differences between the NMR and the thiolysis or HCl-butanol-acetone data. Whilst our results account for a lignin contribution at 155 ppm on the basis of the reference *Tilia* and blackcurrant samples, they do not rule out contributions from other matrix components in the investigated sainfoin plant tissues.[24, 27] Secondly, solid-state NMR experiments are not strictly quantitative because the efficiency of the cross-polarization depends on proton abundance in the neighboring environment, the molecular dynamics and the relaxation behavior for improving the ^{13}C signal.[25, 28] These factors are difficult to fully control in NMR experiments and are unlikely to be the same for all carbons, and will also vary between different plant matrices. Therefore, it is possible that the proton environment of the more complex and possibly larger sainfoin PAs (mDP of 12 to 27) could have resulted in a larger signal enhancement (and consequently PA content) compared to the purified *Tilia* PAs (mDP of 8), but it is unlikely that this factor alone increased the NMR signal by 100% (Table 1).

Another explanation could arise from the limitations of the thiolysis and HCl-butanol-acetone assays in quantitating PAs directly in plant tissues.[3, 4] It has been shown that longer reaction times during thiolysis with benzyl mercaptan resulted in considerably higher concentrations.[5] In addition, the existence of thiolysis-resistant PAs has also been reported.[9] The previous versions of the HCl-butanol assay often underestimated total PA content when applied directly to plant or extracted residue samples, possibly because of incomplete interaction with bound PAs.[12] However, the present HCl-butanol-acetone assay used a recent modification by Grabber et al.,[22] who demonstrated that inclusion of acetone achieved complete degradation of unextractable PAs from two *Lotus* species. Nevertheless, it is possible that sainfoin may have more diverse PA mixtures than *Lotus*,[48] which could explain

the observed differences between the NMR, thiolysis and HCl-butanol-acetone results. Overall, the important point here is that the NMR results suggested that NMR allows accounting for all extractable and unextractable PAs, whereas the thiolysis and HCl-butanol-acetone assays may be underestimating PA contents.

Estimation of Mean Degree of Polymerization. The mean degree of polymerization (mDP) can be obtained by integrating the C3 signals from PA extension units at 73 ppm and the corresponding signals of PA terminal units at 67 ppm in solution NMR.[20, 21, 26] However, significant signal overlap from various plant constituents in milled plant tissues[26] and consequently poor resolution of that region in the ^{13}C CPMAS NMR spectra (Figure 3) prevented estimation of mDP values.

Estimation of procyanidin/prodelphinidin ratios. The procedure to estimate PC to PD ratios within PAs was based on their different hydroxylation patterns, which were clearly distinguishable in the 140-100 ppm region of the ^{13}C CPMAS NMR spectra (Figure 2B). The resonance at 132 ppm was assigned to C1′ and C4′carbons of PDs from blackcurrant, but in *Tilia* PCs this resonance was assigned only to C1′ carbon and had only half the intensity of the PDs signal.[44, 45] This difference in the 132 ppm signal intensity directly reflected the PD and PC composition because the PA contents were comparable (i.e. blackcurrant 87 g PAs/100 g of purified PA fraction; and *Tilia* 95 g PAs/100 g of purified PA fraction); the molar percentages obtained by thiolysis were 94.9% PDs and 97.4% PCs for the purified PA fractions, from blackcurrant and *Tilia* respectively.

This observation was validated by thiolysis and ^{1}H-^{13}C HSQC solution NMR experiments on artificial samples with the nominal PC/PD values equal to 100/0, 70/30,

391 50/50, 30/70 and 0/100, prepared by mixing purified PA fractions from *Tilia* and
392 blackcurrant.

393 However, the solution NMR methods, such as used by Czochanska et al.[20] and Zeller
394 et al.,[23] to estimate PC/PD ratios, which rely on the high resolution of the solution NMR
395 spectra could not be transferred directly to the solid-state NMR experiments. The
396 experimental resolution of the recorded solid-state NMR spectra was in the order of 12.5 Hz
397 (or 0.1 ppm) but the typical linewidth was ca 550 Hz (or 4.5 ppm), as expected for large
398 polymeric molecules. In addition to that, the spectra of milled plant tissues show significant
399 overlap with other signals from the plant matrix below 110 ppm, which prevents any
400 meaningful PC/PD analysis (Figures 3 and 5A). Therefore, it was impossible to calculate
401 PC/PD ratio on the basis of the C2′ - C6′ carbons as used in solution NMR experiments.

402 The experiment on the artificial mixtures of purified PA fractions with nominal
403 PC/PD values was repeated using the solid-state NMR technique. The intensity ratios
404 between signals at 132 and 155 ppm were selected as a calibration standard (to minimize the
405 experimental errors) because they were expected to vary linearly between a value of 1/3 or ca
406 0.33 for pure PCs, and 2/3 or ca 0.67 for pure PDs reflecting the number of carbons
407 contributing to each signal (Figures 2B and 5A). The resulting I_{132}/I_{155} experimental ratios
408 were within the theoretical limits (Figure 5B) and showed excellent linear dependence when
409 plotted as a function of the nominal PC/PD values.

410 However, to extend the procedure to estimate the unknown PC/PD ratios of milled
411 sainfoin tissues, it was necessary to repeat the experiment on the artificial mixtures but this
412 time these were prepared on a plant weight (mg) basis using the milled *Tilia* and blackcurrant
413 tissues. This allowed us to assess and overcome several problems, such as imprecisions in the
414 nominal PC/PD values and plant matrix interference, which varied between different plants
415 and could obscure any direct calculation of the unknown PC/PD ratio using only milled *Tilia*

or blackcurrant plant tissues. It was assumed that the nominal PC/PD values (again equal to 100/0, 70/30, 50/50, 30/70 and 0/100) in these plant tissues would be the same as in the purified PA fraction mixtures. A similar linear dependence of the I_{132}/I_{155} on the nominal PC/PD values was indeed observed for the milled plant tissues compared to the purified PA fraction mixtures (Figure 5B). The large offset between both curves shows that there is a possible interference of the plant matrix. However, both curves showed a similar slope, which confirmed that extraction and purification had no effect on the PC/PD ratio in *Tilia* and blackcurrant mixtures, and that the matrix effect on intensity was almost uniform across these PC/PD ratios. Therefore, the gradient of the I_{132}/I_{155} intensity ratio as a function of nominal PC/PD values could be regarded as a universal tool for setting up a calibration curve to estimate unknown PC/PD ratios in other plant samples such as sainfoin.

Table 1 shows a comparison between the PC content in sainfoin samples from the ^{13}C CPMAS NMR spectra and the thiolysis data. It can be seen that NMR estimated much higher PC contents in purified PA fractions than thiolysis in four of the eight samples, but had a rather low accuracy with an estimated experimental error of ±16 mg PCs/100 mg purified PA fraction. The biggest contributor to the estimated error was the uncertainty in the fitting of the 132 ppm signal, which overlapped with other signals from the plant matrices (Figures 4 and 6A). This was not surprising given the large offset already observed for the I_{132}/I_{155} intensity ratio dependence on nominal PC/PD values between purified PA fractions and milled plant tissues (Figure 5B). However, the degree and pattern of overlap was much larger and variable in the sainfoin compared to the reference *Tilia* and blackcurrant plant tissues (Figure 5A). This problem was particularly evident when comparing the NMR spectra of the S3 and S1 tissues (Figure 4); and was also highlighted in Figure 6, which compared the NMR spectra of sainfoin leaves and stems. Obviously, it was only possible in the case of the fairly resolved structure to extract the correct intensity of the signal at 132 ppm from the spectrum.

Therefore, a better resolution of the NMR spectra will be required to improve the accuracy of the estimated PC molar percentage or PC/PD ratio beyond a qualitative assessment.

However, the comparison of sainfoin leaf and stem spectra also demonstrated the potential of the ^{13}C CPMAS NMR approach to gain a rapid qualitative assessment (Figure 6). The stem spectra showed a very different pattern of resonances in the region between 160 - 120 ppm. This was most likely due to poorer resolution of signals at 144 and 132 ppm in stems, which tend to have higher lignin content than leaves, and also corresponded to lower PA content in stems than leaves[37] and a greater contribution of plant matrix interferences.

This study reported a first attempt to develop a procedure based on ^{13}C CPMAS NMR technique for the direct screening of PAs in sainfoin plant tissues. By using contrasting signature PA spectra from blackcurrant and *Tilia* reference samples, a calibration for PAs was developed based on signal intensities at 155 and 132 ppm. There were surprisingly large differences between the PA content obtained by the thiolysis or HCl-butanol-acetone assays and ^{13}C CPMAS NMR results. These differences could be due to interfering plant matrix components, size and mobility differences in reference and sainfoin PAs, and/or presence of unextractable PAs in sainfoin plant tissues. It is very unlikely that various sizes and mobility differences would influence the cross-polarization enhancement to such an extent that it could account for the observed PA content differences. A simple correction to account for the matrix interference based on the linewidth difference between the purified PA fraction and milled plant tissue has been used. However, even with the applied correction the differences between the ^{13}C CPMAS NMR and thiolysis or HCl-butanol assays indicated that the two degradation methods may not detect the entire PA present in some plants. Therefore, only ^{13}C CPMAS NMR provided full information on both extractable and unextractable PAs in sainfoin plant tissues.

In conclusion, this newly developed procedure based on ^{13}C CPMAS NMR technique has proved to be a useful tool for estimating the upper limit of PA content directly in whole plants and could be of interest for probing the bioactivities of unextractable PAs.

ABBREVIATIONS USED: PA, proanthocyanidin; PC, procyanidin; PD, Prodelphinidin; ^{13}C CPMAS NMR, carbon-13 cross-polarization magic-angle spinning nuclear magnetic resonance; mDP, mean degree of polymerization;

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SUPPORTING INFORMATION DESCRIPTION: ^{1}H-^{13}C Heteronuclear Single Quantum Coherence (HSQC) Nuclear Magnetic Resonance Analysis of the Purified PA Fraction Mixtures; Chromatogram of the Thiolysis Reaction Products from the Blackcurrant Purified PA Fraction; Chromatogram of the Thiolysis Reaction Products from the *Tilia* Purified PA Fraction.

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FIGURE CAPTIONS:

Figure 1. A generic proanthocyanidin structure depicting flavan-3-ol subunits.

Figure 2. (A) Schematic representation of a proanthocyanidin structure consisting of a prodelphinidin subunit (top) and a procyanidin subunit (bottom), and (B) ^{13}C CPMAS NMR spectra (black solid line) of purified prodelphinidin fraction from blackcurrant leaves (upper) and purified procyanidin fraction from *Tilia* flowers (lower). The assignment of the resonances is consistent with the labelled carbon positions of the proanthocyanidin structure including resolved terminal (4t) and internal (4i) carbons. The grey lines depict the corresponding dipolar dephased solid-state NMR spectra, which were optimized to detect only non-protonated carbons. The asterisks mark the spinning side-bands.

Figure 3. Comparison of ^{13}C CPMAS NMR spectra of milled plant tissues (black line), acetone/water crude extracts (green line) and purified proanthocyanidin fractions (blue line) from (A) blackcurrant leaves and (B) *Tilia* flowers. Black arrows depict the signals at 155 and 132 ppm. All spectra were normalized to the amplitude of the 155 ppm signal to evaluate interferences from other plant components in the area of interest (160-120 ppm, highlighted in grey color).

Figure 4. Comparison of ^{13}C CPMAS NMR spectra of sainfoin plant (A) S3, (B) S1 and (C) S4 tissues. The insets show superimposed regions for comparison of the 155 and 145 ppm proanthocyanidin signals and the 53 ppm lignin signal.

Figure 5. (A) Comparison of the selected regions of ^{13}C CPMAS NMR spectra recorded for mixtures with nominal PC/PD values of 100/0, 70/30, 50/50, 30/70 and 0/100, prepared by mixing purified PA fractions (bottom) and milled plant tissues (top) of *Tilia* (PC-rich) and blackcurrant (PD-rich). The red arrows reflect the change in the 132 ppm signal intensity as function of the nominal PC/PD value and a small change in chemical shift. The increase in the color intensity of lines (light grey to black) denotes the increase in the PC/PD value. The black arrows depict other signals that also reflect spectral differences but are less useful due to signal overlaps. All signals were normalized to the amplitude of the signal at 155 ppm (red dot). (B) The relation between the intensity ratio I_{132}/I_{155} and nominal PC/PD values for the calibration mixtures of purified PA fractions and milled plant tissues. The dots denote the experimental points and the red lines show fitted linear dependence.

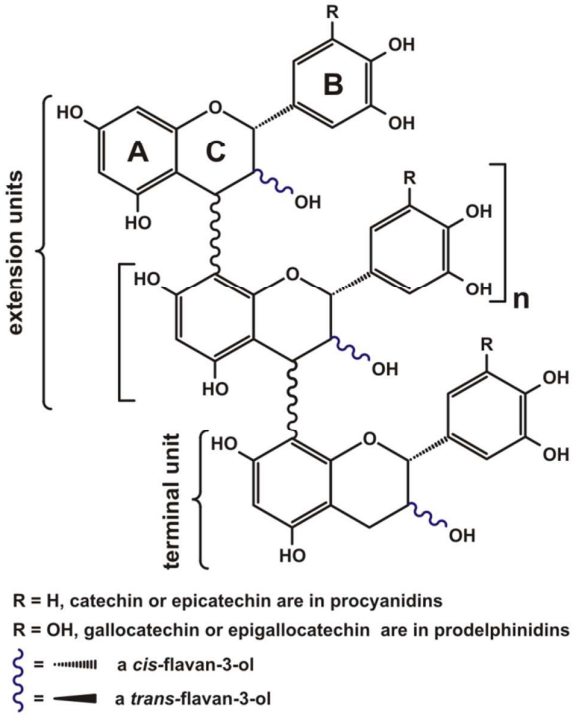
Figure 6. Fingerprint ^{13}C CPMAS NMR spectra from sainfoin (A) leaves and (B) stems. Both spectra were fitted using the sum of Gaussian shape lines and the result of the fit is superimposed on the original spectrum (red dotted lines). The grey lines below show Gaussian lines used to fit the corresponding region of the spectrum. The signals from this region were used to estimate PA content and PC/PD ratios and are shown in blue.

Table 1. Proanthocyanidin (PA) Contents and Procyanidin (PC) Contents within Purified PA Fraction Determined by ^{13}C CPMAS NMR, the HCl-Butanol-Acetone Assay and Thiolysis-HPLC Analysis.

Sainfoin plant tissue	Accession	PA (g/100 g of plant tissue)				PC (mg/100 mg of purified PA fraction)	
		NMR	NMR (– 25%)[a]	HCl-butanol	Thiolysis	NMR	Thiolysis
S1	Zeus	16[b]	12	5.0[c]	1.5[d]	20[b]	23[d]
S2	Zeus	16	12	5.3	3.4	30	18
S3	Cholderton	21	16	8.8	10.0	50	19
S4	Hampshire	14	10	7.1	5.2	30	19
S5	Ambra	18	14	*nt*[d]	5.2	30	19
S6	Zeus	15	11	4.3	1.7	10	18
S7	Perly	14	11	5.1	0.5	20	18
S8[e]	Cotswold Common	19	14	*nt*	4.2	20	19

[a] Correction for lignin interference; [b] experimental error was ± 8 g/100 g of plant tissue for the PA content, and ± 16 mg PCs/100 mg of purified PA fraction for the PC content within purified PA fraction; [c] experimental error was ± 0.5 g/100 g of plant tissue for the PA content; [d] experimental error was ± 0.6 g/100 g of plant tissue for the PA content, and ± 5 mg PCs/100 mg of purified PA fraction for the PC content within purified PA fraction; [d] *nt*: not tested; [e] sample S8 was analyzed only once.

Figure 1.



R = H, catechin or epicatechin are in procyanidins

R = OH, gallocatechin or epigallocatechin are in prodelphinidins

= a *cis*-flavan-3-ol

= a *trans*-flavan-3-ol

Figure 2.

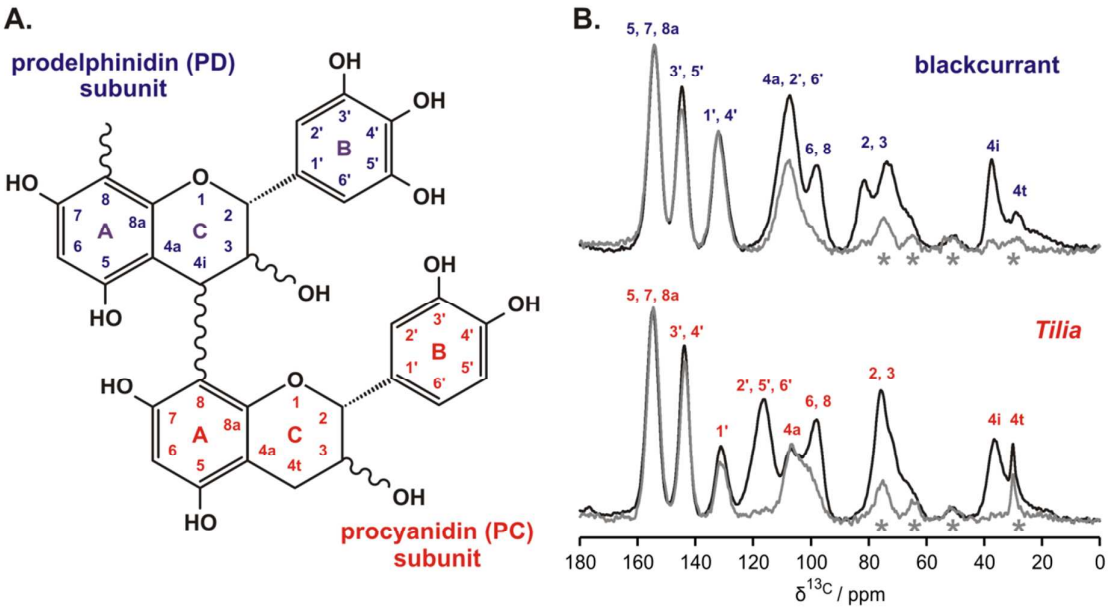

A.
prodelphinidin (PD) subunit
procyanidin (PC) subunit
B.
blackcurrant
Tilia
5, 7, 8a
3', 5'
1', 4'
4a, 2', 6'
6, 8
2, 3
4i
4t
2', 5', 6'
3', 4'
1'
4a
180 160 140 120 100 80 60 40 20 0
δ13C / ppm

Figure 3.

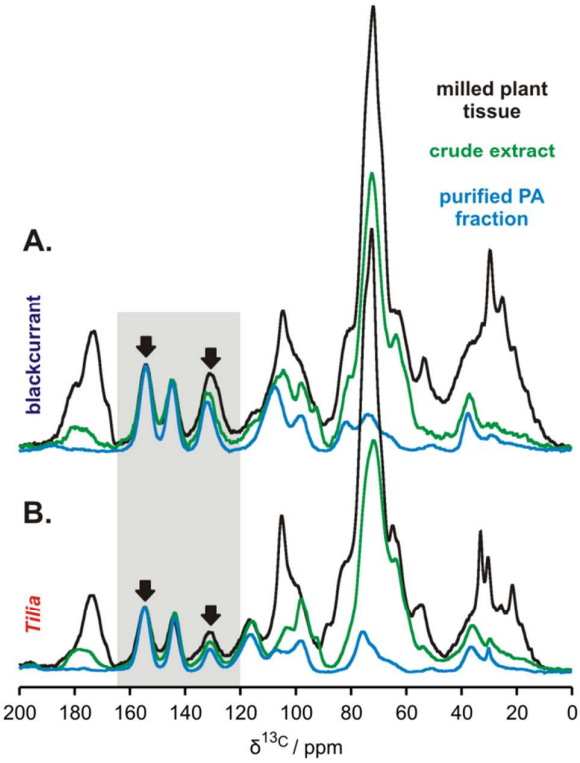

milled plant tissue
crude extract
purified PA fraction
A.
blackcurrant
B.
Tilia
200 180 160 140 120 100 80 60 40 20 0
δ13C / ppm

Figure 4.

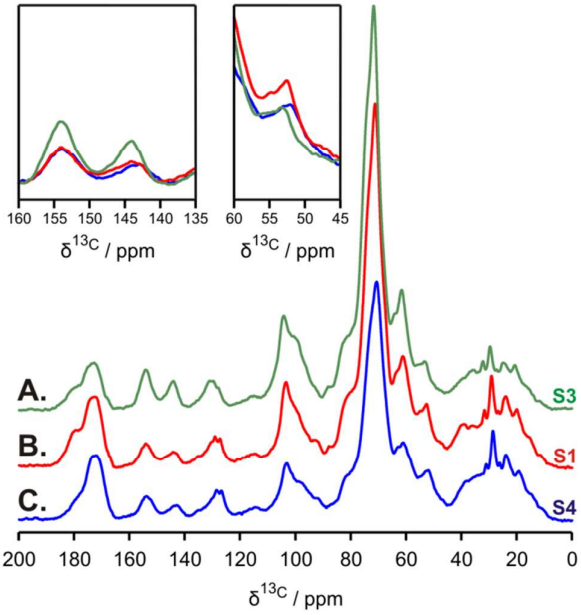

160 155 150 145 140 135
δ13C / ppm
60 55 50 45
δ13C / ppm
A.
B.
C.
S3
S1
S4
200 180 160 140 120 100 80 60 40 20 0
δ13C / ppm

Figure 5.

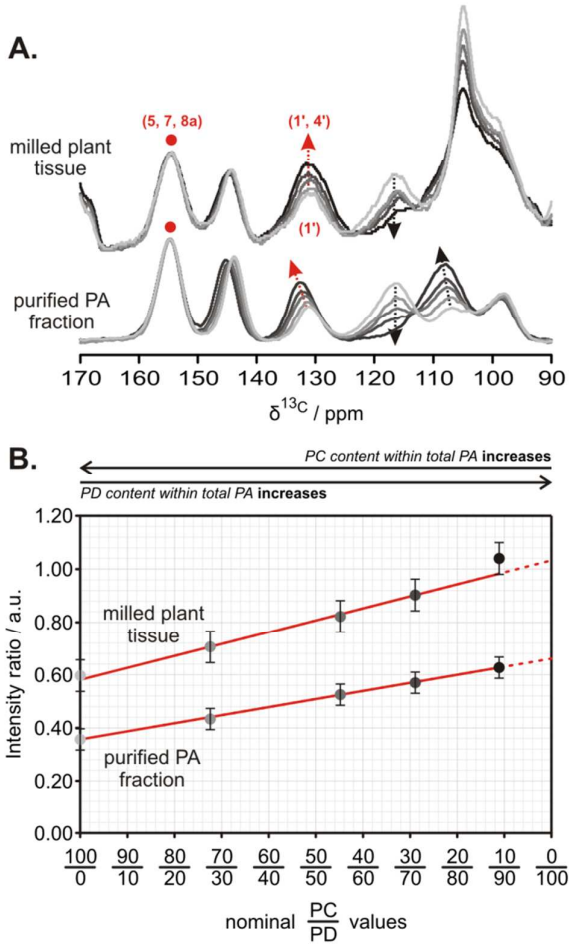
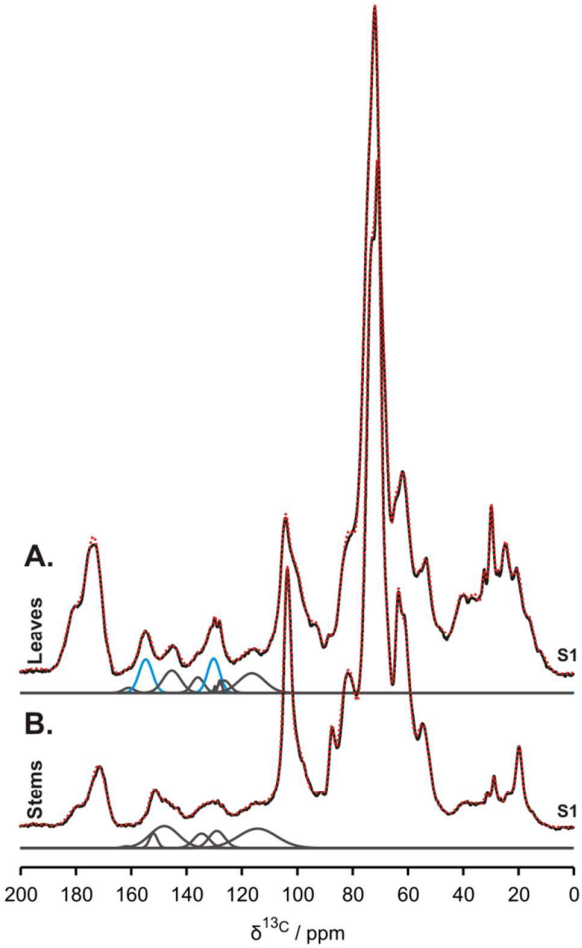

A.
(5, 7, 8a)
(1', 4')
(1')
milled plant tissue
purified PA fraction
170 160 150 140 130 120 110 100 90
δ13C / ppm
B.
PC content within total PA increases
PD content within total PA increases
Intensity ratio / a.u.
1.20
1.00
0.80
0.60
0.40
0.20
0.00
milled plant tissue
purified PA fraction
100/0 90/10 80/20 70/30 60/40 50/50 40/60 30/70 20/80 10/90 0/100
nominal PC/PD values

Figure 6.


A.
Leaves
S1
B.
Stems
S1
200 180 160 140 120 100 80 60 40 20 0
δ13C / ppm

GRAPHIC FOR TABLE OF CONTENTS: