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Quantitative Analysis of Amygdalin and Prunasin in *Prunus serotina* Ehrh. using ^{1}H-NMR Spectroscopy

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ABSTRACT:

Introduction – *Prunus serotina* is native to North America but has been invasively introduced in Europe since the seventeenth century. This plant contains cyanogenic glycosides that are believed to be related to its success as an invasive plant. For these compounds, chromatographic- or spectrometric-based (targeting on HCN hydrolysis) methods of analysis have been employed so far. However, the conventional methods require tedious preparation steps and a long measuring time.

Objective – To develop a fast and simple method to quantify the cyanogenic glycosides, amygdalin and prunasin in dried *Prunus serotina* leaves without any pre-purification steps using ^{1}H-NMR spectroscopy.

Methods – Extracts of *Prunus serotina* leaves using CH_3OH-d_4 and KH_2PO_4 buffer in D_2O (1:1) were quantitatively analysed for amygdalin and prunasin using ^{1}H-NMR spectroscopy. Different internal standards were evaluated for accuracy and stability. The purity of quantitated ^{1}H-NMR signals was evaluated using several two-dimensional NMR experiments.

Results – Trimethylsilylpropionic acid sodium salt-d_4 proved most suitable as the internal standard for quantitative ^{1}H-NMR analysis. Two-dimensional *J*-resolved NMR was shown to be a useful tool to confirm the structures and to check for possible signal overlapping with the target signals for the quantitation. Twenty-two samples of *P. serotina* were subsequently quantitatively analysed for the cyanogenic glycosides prunasin and amygdalin.

Conclusion – The NMR method offers a fast, high-throughput analysis of cyanogenic glycosides in dried leaves permitting simultaneous quantification and identification of prunasin and amygdalin in *Prunus serotina*.

Keywords: quantitative NMR; amygdalin; cyanogenic glycosides; prunasin; *Prunus serotina*.

Introduction

Prunus serotina Ehrl. is a fast growing tree popularly known as black cherry. It is indigenous to North America and was introduced into Europe in the seventeenth century for timber and ornamental purposes (Starfinger *et al*., 2003). *Prunus serotina* was widely planted during the first half of the twentieth century in Europe (Chabrerie *et al*., 2010). However, it is now considered an aggressive invasive species. It has spread very rapidly covering forest openings with a layer of small trees (Chabrerie *et al*., 2008). Its presence affects the populations of native species and the development of a diverse herb layer (Chabrerie *et al*., 2010). The invasiveness of *P. serotina* might be related to the chemical composition of the plants. Cyanogenic glycosides such as prunasin (**1**) and amygdalin (**2**) (Fig. 1) present in this genus are believed to be related to plant protection against herbivores, pathogens and competitors (Swain *et al*., 1992; Swain and Poulton, 1994; Santamour, 1998). Other compounds reported from this plant species include chlorogenic acid, flavonoid glycosides (Olszewska, 2005a,2005b, 2007) and triterpenes (Biessels *et al*., 1974).

Cyanogenic glycosides are produced by various plants and are potentially toxic to herbivores due to hydrolytic release (spontaneous or enzymatically regulated reactions) of hydrocyanic acid (HCN) (Drochioiu *et al*., 2008). There is a wide range of methods for the analysis of cyanogenic glycosides, most of which involve the spectrometric determination of the HCN released by their hydrolysis (Santamour, 1998; Hughes *et al*., 2003; Drochioiu *et al*., 2008). Several chromatographic methods for intact glycosides, involving LC–UV (Mazza and Cottrell, 2008), LC–MS and GC–MS (Balkon, 1982; Tan *et al*., 2011) have also been described. However, these methods are labour intensive and/or time consuming, for example, requiring long chromatographic runs.

Quantitative ^{1}H-NMR spectroscopy, which is very efficient for the simultaneous detection and identification of several

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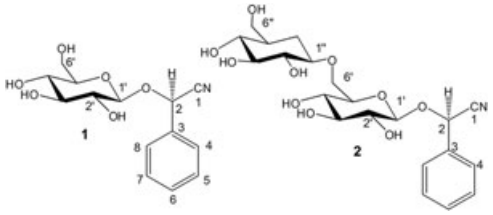

Figure 1. Chemical structures of the cyanogenic glycosides prunasin (**1**) and amygdalin (**2**).

metabolites in crude extracts or samples (Kim *et al*., 2003, 2010; Choi *et al*., 2004; Pauli *et al*., 2005), is a potentially viable alternative method of analysis. As the integrated intensities of the resonance signals are proportional to the molar concentration of the analytes in solution, quantification can be performed with the simple addition of a single internal standard (IS) and subsequent comparison of the integral ratios, without the need of calibration curves for each individual compound.

The objective of the present study was to develop an analytical method to quantify the cyanogenic glycosides amygdalin and prunasin from leaves of a number of individual *P. serotina* trees using ^{1}H-NMR. Resonance signal purity and the identity of the cyanogenic glycosides were confirmed using two-dimensional *J*-resolved, correlated spectroscopy (COSY), heteronuclear multiple bond correlation (HMBC) and diffusion-ordered spectroscopy (DOSY) NMR experiments.

Experimental

Sample collection

Leaves of *P. serotina* were collected in the Nationaal Park Zuid-Kennemerland, The Netherlands on 21 July 2011, from 22 different individual trees (numbered PS01–PS22) ranging from 100 to 1000 cm high. Five young leaves and five old leaves were harvested from each tree, kept on dry ice in the field, and upon return to the laboratory were immersed in liquid nitrogen on the same day. The young and old leaves from each tree were mixed, ground to a fine powder under liquid nitrogen and then freeze-dried to minimise possible metabolite degradation. The 22 powdered samples were then stored in a cold room (−20 °C) in the dark until analysis.

Extraction

Freeze-dried plant material (50 mg) was transferred to a 2 mL microcentrifuge tube. A solution (750 μL) of KH_2PO_4 buffer (90 mM, pH 6.0) in D_2O, containing 0.01% trimethylsilylpropionic acid sodium salt-d_4 (TMSP, w/v), and CH_3OH-d_4 (750 μL) were added to the plant samples. The mixture was vortexed at room temperature for 2 min, ultrasonicated by Branson-3510 Ultrasonic Cleaner (Thomas Scientific, Swedesboro, NJ, USA) at 40 kHz for 15 min at room temperature, and centrifuged at 10968 × *g* for 15 min. An aliquot of the supernatant (800 μL) was transferred to a 5 mm NMR tube. Samples were stored at 4 °C in the dark until analysis. Extractions and subsequent NMR measurements were performed in duplicate for all samples except samples PS1, PS3, PS4, PS6, PS9, PS12 and PS18, which were carried out in triplicate.

Two-dimensional DOSY sample preparation

For the two-dimensional DOSY experiment methanol (500 μL) and water (500 μL) were added to PS10 (50 mg) for extraction as described above. The supernatant was decanted into a 2 mL microcentrifuge tube, methanol was removed in speed vacuum and the remaining H_2O freeze-dried. The lyophilisate was resuspended in DMSO-d_6 (800 μL) for NMR acquisition.

Internal standards

To test internal standards, PS1 (30 mg) was extracted with CH_3OH-d_4 (750 μL) and a solution of KH_2PO_4 buffer in D_2O (90 mM, pH 6.0) (750 μL), containing 0.01% (w/v) TMSP and 10% (w/v) of maleic acid as internal standard. An aliquot (800 mL) of the supernatant was transferred to a 5 mm NMR tube. Solutions of 3,4,5-trichloropyridine (5.3 mg) in CH_3OH-d_4 (5 mL) and 1,4-dinitrobenzene (5.1 mg) in CH_3OH-d_4 (5 mL) were prepared. Two additional samples of PS1 (30 mg) were extracted separately using 150 μL of 3,4,5-trichloropyridine solution in one of the samples and 150 μL of 1,4-dinitrobenzene solution being added to the other. CH_3OH-d_4 (600 μL) and KH_2PO_4 buffer in D_2O (90 mM, pH 6.0) (750 μL), containing 0.01% (w/v) TMSP were added to both samples. An aliquot (800 mL) of each supernatant was transferred to a 5 mm NMR tube.

Amygdalin (3.31 mg) was added to a microcentrifuge tube to which was added CH_3OH-d_4 (450 μL), the IS solutions (150 μL of each) and KH_2PO_4 buffer in D_2O (90 mM, pH 6.0) (750 μL), containing 0.01% (w/w) TMSP. An aliquot of the solution (800 μL) was transferred to a 5 mm NMR tube.

The NMR experiment

The one-dimensional (^{1}H) and two-dimensional (^{1}H-^{1}H) *J*-resolved NMR spectra for the quantitative analysis were recorded at 25 °C on a 500 MHz Bruker DMX-500 spectrometer (Bruker, Karlsruhe, Germany) operating at a proton NMR frequency of 500.13 MHz. Methanol-d_4 was used as the internal lock. Each ^{1}H-NMR spectrum consisted of 128 scans, with 3.17 s of acquisition time with the following parameters: 0.16 Hz/point, pulse width = 30° (11.3 ms) and relaxation delay = 15 s. A pre-saturation sequence was used to suppress the residual H_2O signal with low-power selective irradiation at the H_2O frequency during the recycle delay. Free induction decays were Fourier-transformed with line broadening = 0.3 Hz. The resulting spectra were manually phased, baseline-corrected and calibrated to TMSP at 0.00 ppm using MestReNova NMR suite (Version 7.02) program obtained from Mestrelab Research S.L. (Santiago de Compostela, Spain). ^{1}H-^{1}H *J*-resolved NMR spectra were acquired with pre-saturation (gB1 = 50 Hz) during a relaxation delay of 1.5 s. The spectra were recorded using eight scans per 128 increments for F1 and 8192 for F2, with spectral widths of 5000 Hz in F2 (chemical shift axis) and 66 Hz in F1 (spin–spin coupling constant axis). Datasets were zero-filled to 512 points in F1 and both dimensions were multiplied by sine-bell functions (SSB = 0) prior to double complex FT. J-resolved spectra tilted by 45°, were symmetrised about F1, and then calibrated, using XWIN NMR (Version 3.5, Bruker).

^{1}H-^{1}H COSY spectra were acquired with pre-saturation (gB1 = 50 Hz) during a relaxation delay of 1.5 s. A data matrix of 4096 (F2) × 512 (F1) points covering 5000 × 5000 Hz was recorded with eight scans for each increment. A sine bell-shaped window function (SSB = 0) was applied in both dimensions. For HMBC spectra a data matrix of 4096 (F2 axis) × 512 (F1 axis) points covering 7002 × 26411 Hz was recorded with 32 scans for each increment. A relaxation delay of 1.5 s and a coherence transfer delay optimised for a long-range coupling of 8 Hz were applied. Data were linear-predicted to 1024 points using 32 coefficients prior to QF type two-dimensional Fourier transformation and a sine bell-shaped window function (SSB = 0) was applied. The final spectrum was obtained by magnitude calculation along the F2 dimension. The two-dimensional NMR files were processed and analysed with the MestReNova NMR.

The DOSY NMR experiments were performed on a Bruker Avance 400 MHz spectrometer with a 5 mm *z*-gradient inverse probe. The sample solvent, DMSO-d_6, provided the deuterium lock and the internal spectrum reference. The NMR spectrum was acquired with a spectral width of 5580.4 and (gradient strength) gradient pulse amplitudes ranging from 95% to 1%. Data processing analysis of the DOSY data was performed by the MestReNova NMR suite.

Sensitivity and reproducibility of the NMR method

The sensitivity was evaluated by measuring the minimum concentration of the analyte that could be detected above an S:N ratio of 10 with the number of scans fixed at 128. Five samples containing increasing amounts of amygdalin (6.0, 13.5, 27.0, 75.0 and 150.0 μg/mL) in methanol:KH_2PO_4 buffer (90 mM, pH 6.0) in D_2O containing 0.01% TMSP (1:1) were prepared and the ^{1}H-NMR spectrum of each was recorded. The integrals were measured and the quantity of the amygdalin was determined. The accuracy of the method was evaluated by calculating the recovery of 2.0 μg of amygdalin from a methanolic solution spiked into PS9 freeze-dried leaf material. The solvent was evaporated in speed vacuum and the sample extracted as described in the extraction protocol. The experiment was performed in quadruplicate. Reproducibility was determined by comparing peak areas of each compound in a PS1 extract for both intraday (three measurements) and interday analyses during 3 days.

Results and discussion

The proton NMR spectra of prunasin (**1**) and amygdalin (**2**) in different solvents are well documented (Horsley and Meinwald, 1981; Ribeiro, 1990; Nahrstedt and Rockenbach, 1993). The methine proton of the CHCN moiety resonates as a sharp singlet around δ 5.90 for prunasin and δ 5.86 for amygdalin in a non-crowded region of the ^{1}H-NMR spectra of *Prunus* crude extracts.

Two basic requirements for an internal standard (IS) should be met, that is, the signal of the IS and the target signal from the analyte should not overlap, and the resonance of the IS and the selected compound should not occur in crowded spectral regions. The suitability of various compounds such as maleic acid, 3,4,5-trichloropyridine and 1,4-dinitrobenzene, were tested at a final concentration of 0.5 mg/mL in the extraction solvent. Maleic acid, which has been used for the NMR analysis of crude extracts (Choi *et al*., 2003; Frédérich *et al*., 2003), proved unsuitable in this study because its signals overlapped with some signals present in the *P. serotina* spectra. The 3,4,5-trichloropyridine was discarded due to handling difficulties (e.g. highly hygroscopic). On the other hand, 1,4-dinitrobenzene is available in high purity, is inexpensive, can be easily weighed, and has been reported as a suitable internal standard, providing a sharp signal at δ 8.50, a usually non-crowded spectral region (Molyneux *et al*., 1979; Rundlöf *et al*., 2010). However, our experiments showed that the compound was not stable enough for quantitative metabolomic analysis. In methanol-d_4/ KH_2PO_4 buffer (90 mM, pH 6.0) in D_2O containing 0.01% TMSP solution (1:1) the 1,4-dinitrobenzene signal clearly decreased after a period of 11 h, owing to the exchange of protons against deuterium in the solvent. Storage of samples with 1,4-dinitrobenzene would therefore lead to erroneous results. This was confirmed by the higher variability of the results obtained using 1,4-dinitrobenzene as the IS (RSD% 11.1) than those obtained using TMSP (RSD% 3.0). Therefore, TMSP was chosen as an IS for the analytical protocol of *P. serotina* extracts.

A rather long relaxation delay of 15 s was chosen for the NMR experiments in order to avoid saturation (Rundlöf *et al*., 2010). The LOQ was determined to be 13.5 mg/mL, the recovery of spiked amygdalin was above 98.65% ± 4.28 (±SD, $n = 4$), and the RSD for prunasin was 0.4% and 2.92% for amygdalin in intraday experiments and 1.67% and 2.99% for prunasin and amygdalin, respectively, for the interday experiments.

To confirm the presence and identities of specific components in a complex mixture, different 2D NMR methods (*J*-resolved, COSY, HMBC, DOSY) can be employed. In this case, a DOSY experiment on plant sample PS10, in two different solvents, that is, the solvent used for the quantification experiments CH_3OH-d_4-KH_2PO_4 in D_2O at pH 6.0, and DMSO-d_6 did not fully resolve the signals of the cyanogenic glycosides to allow full structure confirmation. Thus, ^{1}H-^{1}H *J*-resolved and HMBC spectra were used for the identification of cyanogenic glycosides in the plants. In a ^{1}H-NMR spectrum of a mixture, many signals of diverse metabolites are likely to overlapwith each other. ^{1}H-^{1}H *J*-resolved spectra in which the chemical shift and spin– spin coupling data onto different axes have been shown to be effective for the investigation of signal purity in a congested mixture of multiplets. Two singlets belonging to prunasin and amygdalin, respectively were well separated in ^{1}H-^{1}H *J*-resolved spectrum (Fig. 2A). As the next step of identification of two cyanogenic glycosides, a HMBC spectrum was used to obtain information on the correlation between H-2 and adjacent carbons. In Fig. 2B H-2 of prunasin and amygdalin were confirmed by HMBC spectrum by clear correlation with C-1, C-3 and C-4/C-8. Using HMBC it was possible to distinguish H-2 of prunasin and

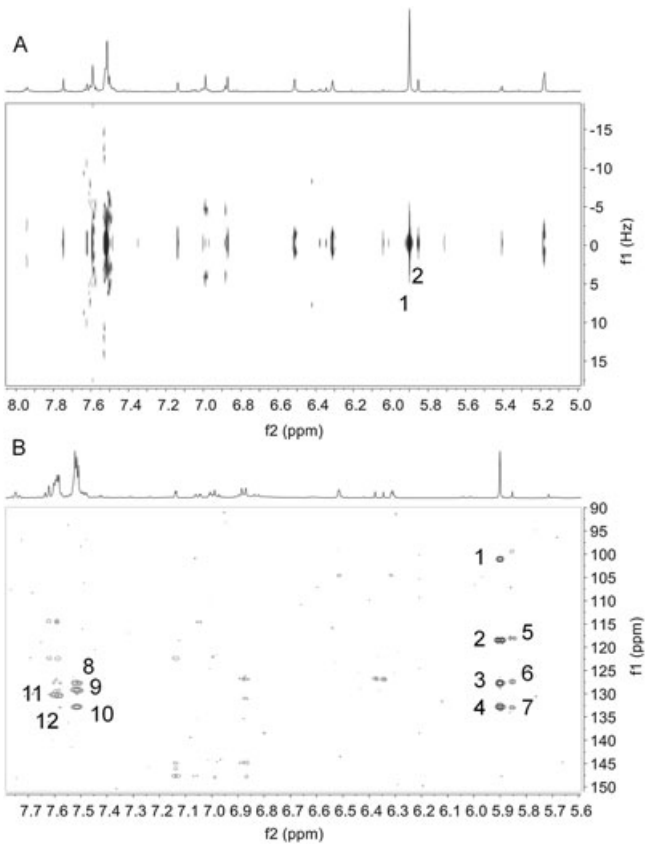

Figure 2. Typical ^{1}H-^{1}H (A) *J*-resolved and (B) HMBC spectra of *Prunus serotina* leaves (CH_3OH-d_4-KH_2PO_4 in D_2O at pH 6.0 extract) in the range of δ 5.1–7.7 for ^{1}H-NMR and δ 90–170 for ^{13}C-NMR: (**1**) prunasin and (**2**) amygdalin. In the HMBC spectra (B): 1, correlation of H-2 and C-1′ of prunasin; 2, correlation of H-2 and C-1 of prunasin; 3, correlation of H-2 and C-4 of prunasin; 4, correlation of H-2 and C-3 of prunasin; 5, correlation of H-2 and C-1 of amygdalin; 6, correlation of H-2 and C-4 of amygdalin; 7, correlation of H-2 and C-3 of amygdalin; 8, correlation of H-5/H-7 and C-4; 9, correlation of H-6 and C-4/C-8; 10, correlation of H-5/H-7 and C-3; 11, correlation of H-4/H-8 and C-5/C-7 of prunasin; 12, correlation of H-4/H-8 and C-5/C-7 of amygdalin.

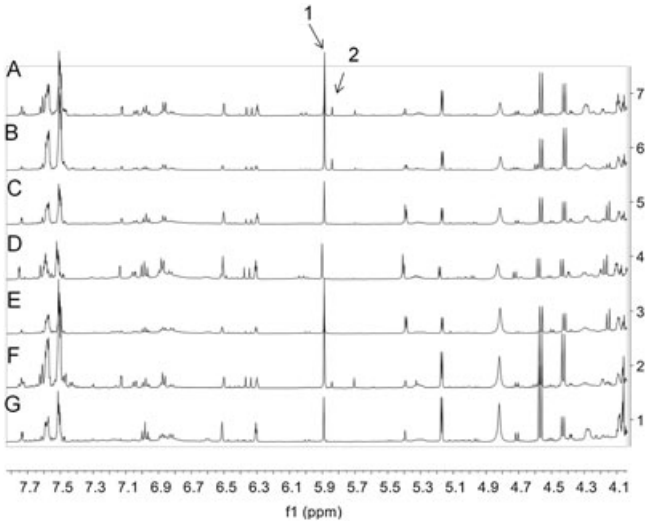

Figure 3. The ^{1}H-NMR spectra (methanol-d_4-KH_2PO_4 in D_2O at pH 6.0 extract) of *P. serotina* leaves of (A) PS01, (B) PS03, (C) PS04, (D) PS06, (E) PS09, (F) PS12, and (G) PS18 in the range of δ 4.0–7.8: (1) H-2 of prunasin, (2) H-2 of amygdalin.

Table 1. Content of prunasin and amygdalin in leaf extracts (mg/g dry weight ± SD) of *Prunus serotina* (quantified by their ^{1}H-NMR spectrum)

Samples	Prunasin		Amygdalin	
	mg/g ± SD	RSD	mg/g ± SD	RSD
PS 01[a]	33.99 ± 1.52	4.46	7.40 ± 0.41	5.59
PS 02	43.47 ± 0.33	0.75	20.95 ± 0.25	1.21
PS 03[a]	56.42 ± 4.84	8.59	11.10 ± 0.95	8.54
PS 04[a]	18.19 ± 1.09	6.01	Tr	NC
PS 05	28.09 ± 1.14	4.07	3.76 ± 0.76	20.20
PS 06	15.11 ± 0.35	2.34	Tr	NC
PS 07	28.09 ± 0.16	0,58	4.300 ± 0.000	0.000
PS 08	10.17 ± 0.33	3.21	2.15 ± 0.000	0.000
PS 09[a]	17.19 ± 1.16	6.77	Tr	NC
PS 10	59.49 ± 1.31	2.20	12.90 ± 0.000	0.000
PS 11	15.38 ± 1.47	9.57	Tr	0.000
PS 12[a]	45.78 ± 2.08	4.55	4.42 ± 0.21	4.68
PS 13	17.80 ± 0.33	1.84	Tr	NC
PS 14	30.64 ± 0.16	0.53	Tr	NC
PS 15	25.20 ± 0.33	1.300	4.48 ± 0.25	5.66
PS 16	20.46 ± 0.49	2.400	Tr	NC
PS 17	26.24 ± 0.16	0.62	Tr	NC
PS 18[a]	9.71 ± 0.46	4.76	Tr	NC
PS 19	36.88 ± 0.16	0.44	Tr	NC
PS 20	20.58 ± 0.65	3.18	Tr	NC
PS 21	18.38 ± 0.82	4.45	0.72 ± 0.000	0.000
PS 22	17.11 ± 0.00	0.00	2.15 ± 0.000	0.000

[a]Values shown are means (± SD) based on triplicate measurements.
Tr, trace levels detected (less than limit of quantitation, defined as signals larger than 10 times S:N); NC, not calculated.

amygdalin, allowing the identification of prunasin in all 22 analysed samples and amygdalin in eight samples.

The prunasin and amygdalin contents of the leaves of 22 different trees of *Prunus serotina* were determined using the developed ^{1}H-NMR method. In all of the 22 spectra, the signals of the characteristic methine protons from prunasin and amygdalin were clearly separated from each other (Fig. 3). The concentration of prunasin was in the range of 9.71 to 59.49 mg/g dry weight, whereas amygdalin was detected in traces and up to 20.95 mg/g dry weight (Table 1).

The method described in this paper is simple, robust, and is suited for the high throughput analysis of a large number of *P. serotina* samples. The method developed can be applied to quantitatively analyse cyanogenic glycosides in *P. serotina* in response to different variables, for example developmental stage, season, diurnal changes, effect of herbivory and diseases, habitat and genotype.

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