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Chromatographic Determination of Cyanoglycosides Prunasin and Amygdalin in Plant Extracts Using a Porous Graphitic Carbon Column

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The determination of cyanogenic compounds in plants is often performed by HPLC. However, in this analysis, interferences due to compounds in the matrix, such as tannins and other pigments, are encountered, especially in roots and leaves. A new method is proposed for determining the cyanogenic glycosides amygdalin (D-mandelonitrile β-D-gentiobioside) and prunasin (D-mandelonitrile β-D-glucoside) in almond tree tissues, using poly(vinylpyrrolidone) or active carbon as scavengers for extracting cyanogenic compounds from roots or leaves, respectively. A new chromatographic approach for conducting the analysis is also discussed herein. The advantages of a Hypercarb column for the analysis of prunasin in roots are shown. The correlation coefficient with a reference method is high ($>$0.99), and statistical tests prove that the two methods are equivalent. In addition, the results provide evidence that prunasin is the only cyanoglycoside present in almond tree roots.

KEYWORDS: Chromatography; amygdalin; prunasin; almond tree tissues

INTRODUCTION

Cyanoglycosides are toxic components found in many plants and are especially present in roots, tubers, seeds, and fruits, which are consumed as foods and feeds in large areas around the world (*1*). Owing to the toxic nature of these cyanogenic compounds, the analytical community has been continually struggling to develop and optimize procedures for their detection and monitoring (*2*). The determination of cyanogenic compounds is performed mainly by HPLC (*3*, *4*), although other analytical methods employ GC or GC−MS after a suitable derivatization (*5*, *6*). Liquid chromatography is normally conducted using reverse-phase C18 columns, acetonitrile:water mixtures as eluent, and detection with UV absorption. Electrochemical detection has also been documented for HPLC (*7*).

The diglucoside amygdalin, the only cyanogenic compound found in seeds, is responsible for the taste of bitter almond seeds (*1*, *8*). In roots, however, monoglucoside prunasin seems to be the only cyanogenic compound present (*7*, *9*), and it has also been found in general in a lower amount in other vegetative tissues of plants.

Amygdalin in seeds is currently determined by reverse-phase liquid chromatography using a C18 column, after extraction of dry samples in methanol. The results can be compared with those of total cyanide obtained after enzymatic hydrolysis, by microdiffusion (*10*) and photometry determination (*11*−*13*) or gravimetric titration (*14*). Nevertheless, extracts of roots or leaves are rich in compounds, such as tannins, flavonoids, and chlorophylls, which elute at the same time as amygdalin in the current chromatographic analysis by HPLC with a C18 column. As a result, this method fails for a complete analysis of both cyanogenic compounds in almond tree tissues.

The aim of our work was to develop a method for the chromatographic identification and determination of both cyanogenic compounds in juvenile trees. This is a requirement in breeding programs as a predictive test for the quality of future seeds.

MATERIALS AND METHODS

Roots, leaves, and seeds were collected from different trees grown in the Experimental Fields of Centro de Edafología y Biología Aplicada del Segura (CEBAS) in Murcia (Spain) for breeding purposes. They had all been well characterized genotypically and were selected to cover a broad range of cyanoglycoside content. The collected samples were lyophilized and stored dry until analysis.

Chromatographically purified β-glucosidase from almonds, prunasin, and amygdalin, used as standars, and poly(vinylpyrrolidone) (PVP-360) were obtained from Sigma-Aldrich (Madrid, Spain) and carbon CNR 115 from Norit N.V. (Amersfoort, The Netherlands). Carbon was activated from time to time at 200 °C for 2 h and kept in a desiccator.

A Waters 600 E system controller, coupled with a photodiode array detector (Waters 996 series), and a Rheodyne 7125 model injector were used. Data were collected and processed by the Waters Millenium 2010 chromatography data system.

A Memmert WB-14 shaking thermostat bath (±0.1 °C) was also employed.

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Cyanoglycoside Extraction. Plant samples of about 0.2−0.4 g were shaken with 10 mL of methanol for 12 h at room temperature in a reciprocal shaker, in the presence of 0.5 g of activated carbon (for leaves) or 0.2 g of poly(vinylpyrrolidone) (for roots). Cyanogenic compounds in seeds were extracted with only methanol in the same way. Mixtures were centrifuged at 3000 rpm for 10 min. Suspensions were subsequently decanted, and the supernatant was filtered through a 0.45 μm nylon filter. Aliquots from this solution were injected directly into the column.

Total cyanide determination. Two steps were involved:

(a) For the release of hydrogen cyanide by enzymatic hydrolysis, 0.2 g of sample was incubated with 0.1 g of β-glucosidase in 4 mL of acetate buffer (pH 5.5) in a small cylindrical glass vessel (3 cm diameter × 5 cm high) for 24 h at 35 °C. The released hydrogen cyanide was collected by microdiffusion into 1 mL of 0.2 M NaOH solution in a smaller glass collector located in the interior of the vessel. Details of this procedure are found elsewhere (*10*).

(b) Measurement of cyanide was carried out by titration or spectrophotometrically depending on the amount of cyanide. Gravimetric titration was performed with a $AgNO_3$ standard solution and 5-(4-dimethylaminobenzilidene) rhodanine indicator, for samples containing more than 20 mg of cyanide/100 g of sample, in accordance with a recommended procedure (*14*). Spectrophotometric determination at 580 nm, after derivatization with barbituric acid in pyridine (*11*), was employed for samples with a lower cyanide content.

Chromatography. Two modes of chromatography were applied:

(a) With a reverse-phase C18 column, the determination was performed isocratically, as described elsewhere (*15*), by a procedure similar to that proposed by Kajiwara and Tomiyama (*3*). Experiments were performed under the following conditions: C18 Symmetry column, Waters (250 × 4.6 mm, 5 μm), Symmetry Guard column (200 × 4 mm), 20 μL of sample, acetonitrile:water (20:80) as eluent at a 1.3 mL/min flow rate, and UV detection at 218 nm.

(b) With porous graphitic carbon, the determination was performed isocratically using a Hypercarb column, ThermoQuest, Hypersil (100 × 4.5 mm, 5 μm), Hypercarb Guard column (10 × 4 mm), a 1.5 mL/min flow rate, methanol:water (90:10) as eluent, 20 μL of sample, and UV detection at 218 nm.

RESULTS AND DISCUSSION

Methanol is an excellent solvent for cyanogenic compounds, and it is thus the solvent of choice for the extraction of amygdalin and prunasin in fruits (*5*). Following the procedure described above, recovery of the cyanoglycosides from seeds, roots, or leaves exceeded 98% for a single extraction with methanol. This was verified by the minute additional amount of cyanoglycosides found (<2%) in a subsequent treatment of the residue with a fresh portion of methanol. Along with cyanoglycosides, green and red pigments were extracted from leaves and roots, respectively; these pigments included chlorophylls, tannins, anthocyanins, and other polar compounds. No other solvent system tested succeeded for a selective extraction of cyanoglycosides.

Activated carbon is known to have important decolorizing properties. With this in mind, the extractions described above were carried out in the presence of activated carbon. The root extracts were only partly decolorized. However, the green pigments were almost eliminated from leaf extracts; thus, interfering peaks disappeared during subsequent chromatograms, and the cyanoglycosides in leaves could be determined in a precise and simple way using the C18 method. Only prunasin was found in leaves, but amygdalin would also have been detected. Regarding this procedure, it is important to use a carbon material of sufficient grain size and mechanical strength, such as that recommended here, to minimize abrasion via shaking during the extraction; otherwise the sample filtration before chromatography would be very difficult.

To clean up the red extracts obtained from roots, other sorbents such as gelatin and insoluble PVP were investigated. The latter reagent has been previously used for the isolation of dhurrin (*16*), and it was recently verified in our laboratories as a selective sorbent for red pigments from almond roots. The purification and analysis was achieved in the same manner as described for the activated carbon. Chromatograms with a C18 column of aliquots from the corresponding filtrates exhibited a clean peak for prunasin close to 6 min, after some resolved peaks, one of which occurred at the same time as the only one found in an amygdalin standard solution (approximately at 3.5 min). The remaining peaks were due either to compounds not removed from sample or to a soluble portion of the commercial PVP itself. Analysis with diode array detection and comparison with total cyanide results showed that the peak at 3.5 min in roots did not correspond to amygdalin. So, our results showed that although PVP can be used to purify samples of components that coelute close to prunasin, this approach was unable to ensure the presence or absence of amygdalin, and therefore prevents its simultaneous determination with the C18 column used.

Columns of porous graphitic carbon have been used with success in the separation of nonpolar and polar compounds in the same sample (*17*), and hydroxylated compounds in particular, as polyphenols and oligosaccharides (*18*). Even very similar substances, such as enantiomers (*19*), and complex mixtures of polychlorinated dibenzo-*p*-dioxins and dibenzofurans have been successfully resolved (*20*). The mechanism of such separations is not well understood; however, structural effects, electronic effects, and the designed "polar effect" of the porous carbon apparently play a role in the selective separations described in the literature (*21*). While the main interactions are hydrophobic, this polar effect could explain the frequent reverse order of elution of similar compounds, in relation to their elution from C18 columns.

Figure 1 shows the chromatograms obtained from the PVP methanolic extracts of the same root sample, in (A) using a C18 column, and in (B) and (C) using an Hypercarb column. In **Figure 1C**, the original sample was spiked with amygdalin, which clearly was not present in the root extracts. Chromatogram A (as all the chromatograms with the C18 column) was recorded with a delay time, since the first stage of the chromatograms proved to have no significance for this analysis. Cross-comparing the chromatograms, the differences between chromatograms were apparent. First, the order of elution of cyanoglycosides was reversed (prunasin elutes at approximately 2 min, and amygdalin later at approximately 7.6 min); second, peaks which interfered with the amygdalin in the C18 columns preceded the peak of the prunasin, enabling the unambiguous determination of both cyanoglycosides. Chromatograms with Hypercarb were lacking certain peaks that were present with a conventional C18 column, apparently due to some components in the samples, which were retained rather strongly in the graphitic precolumn. The baseline deteriorated somehow over the course of a day's work, although without preventing satisfactory results; however, the baseline became again perfectly flat by the following morning if the column was continuously washed overnight with the eluent at a low flow rate (0.1 mL/min). Without taking any other precaution, but changing the precolumn after every 100 analyses, the column continues working satisfactorily after more than 4000 almond tree samples analyzed.

Using a methanol:water (90:10) mixture and a 1.5 mL/min flow rate, retention times of both cyanoglycosides in standard and sample solutions were reproducible (between days, better

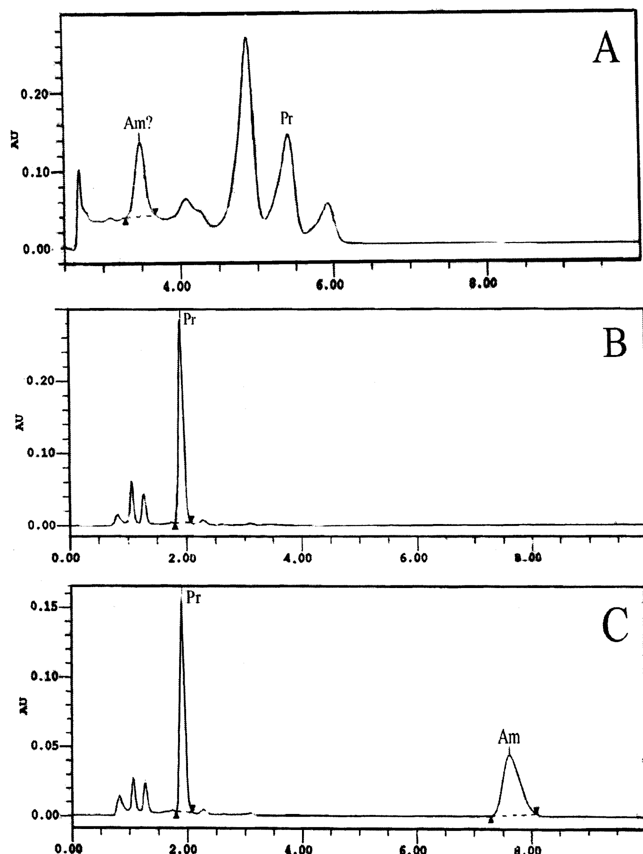

Figure 1. (A) Chromatogram of a root methanolic extract using a C18 column as described in the text: Am, amygdalin; Pr, prunasin. (B) Chromatogram of an extract from the same root sample using a Hypercarb column as described in the text. The only cyanogenic compound found is prunasin. (C) Chromatogram of the same extract as in (B) spiked with amygdalin and using the Hypercarb column.

than 0.2% RSD) and so different that there is no way they would have been mistaken for each other in other mixtures similar to those tested in this study.

Chromatographic determination of cyanoglycosides in seeds and leaves can also be performed using the Hypercarb column method from methanolic extracts of seeds and in the presence of activated carbon in the case of leaves. Using this method, a partial transformation of amygdalin into prunasin or vice versa could be easily monitored.

Analytical results for roots, leaves, and seeds obtained using the Hypercarb column are shown in **Table 1**. They are expressed as cyanide for the sake of comparison with those obtained by the reference method of total cyanide. In roots and leaves no amygdalin is found, while in seeds this is the only cyanogenic detected. Variability of data given in the table ranges from 3% to 5% for the three methods and for samples containing more than 50 mg of cyanide per 100 g of dry sample. It reaches 10% in a few more complex samples analyzed. In these cases, the median seems to be more convenient than the mean value.

The results compare well with those from diffusion followed by gravimetric titration, which are considered as the reference in this work. Specifically, the Pearson correlation coefficient between the two methods is 0.995.

In **Table 1** are also shown the results obtained by applying the C18 method to the same samples. Seemingly, they also correlate rather well with those from the reference method. The values given are computed considering exclusively the prunasin peak, that is, disregarding the artifact appearing at the retention time of amygdalin. Chromatography with a Hypercarb column has, therefore, demonstrated that prunasin is the only cyanoglucoside found in almond tree roots. Moreover, this method has been shown to be advantageous over the C18 method, as it does not induce false positive peaks of amygdalin. The regression equation between the proposed chromatographic method and the titrimetric one is $y = 8.162 + 0.9744x$ ($r^2 = 0.9895$). Statistical t tests reveal only a slight constant bias between the two methods (the possibility that the more complex titrimetric method is defective can be suspected), and the new method has no proportional error at a 5% level of significance.

Table 1. Average Content of Cyanide in Roots (R), Leaves (L), and Seeds (S) of Almond Trees (mg/100 g Dry Basis)

sample	gravimetric titration	chromatography	
		Hypercarb	C18[a]
R1	103	102	94
R2	141	142	157
R3	57	77	63
R4	83	102	95
R5	64	76	78
R6	83	102	95
R7	88	102	92
R8	42	52	48
R9	103	120	100
R10	141	151	134
R11	67	75	78
R12	63	71	63
R13	61	69	77
R14	74	85	84
R15	62	59	76
R16	122	133	114
R17	184	192	162
R18	134	138	140
R19	319	314	361
R20	306	302	260
L1	43	41	42
L2	58	55	54
L3	46	40	42
L4	5	8	8
S1	0,1	<0.1	0,1
S2	6,6	5,8	6,1
S3	208	202	207

[a] In the data for roots using C18, the false peak of amygdalin (peak 1 in **Figure 1A**) is not computed.

The reproducibility of chromatographic areas within the day and between days from root extracts, at a level of prunasin equivalent to 5 ppm CN^-, is estimated to be close to 3% from five independent determinations. The calibration curve for prunasin is found to be linear, at least from 1×10^{-4} to 1×10^{-3} M, and can be adjusted to the equation

$$y \text{ (peak area)} = (3.5 \times 10^9)x \text{ (M)} + (8.5 \times 10^4)$$

whereby the detection limit for cyanide is calculated to be 3 mg of CN^-/100 g of dry root. Similar results have been verified for amygdalin in seeds.

In conclusion, chromatographic separation and determination of prunasin and amygdalin in extracts from almond tree samples can be effectively achieved. Simultaneous determination of both cyanoglycosides in the same sample could contribute to the understanding of their metabolism in the vegetative cycle of almond trees. We expect that this methodology could also be used for the determination of these cyanoglycosides present in samples from other plants.

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LITERATURE CITED

(1) Conn, E. E. Cyanogenic compounds. *Annu. Rev. Plant Physiol.* **1980**, *31*, 433−451.
(2) Yeoh, H.; Tatsuma, T.; Oyama, N. Monitoring the cyanogenic potential of cassava: the trend towards biosensor development. *Trends Anal. Chem.* **1998**, *17* (4), 234−240.
(3) Kajiwara, N.; Tomiyama, C. Determination of amygdalin in apricot kernel and processed apricot products by HPLC. *J. Food Hyg. Soc. Jpn.* **1983**, *24* (1), 42−46.
(4) Gómez, E.; Burgos, L.; Soriano, C.; Marín, J. Amygdalin content in the seeds of several apricot cultivars. *J. Sci. Food Agric.* **1998**, *77*, 184−186.
(5) Frehner, M.; Scalet, M.; Conn, E. E. Pattern of the cyanide-potential in developing fruits. *Plant Physiol.* **1990**, *94*, 28−34.
(6) Chassagne, D.; Crouzet, J. C.; Bayonove, C. L.; Baumes, R. L. Identification and quantification of passion fruit cyanogenic glucosides. *J. Agric. Food Chem.* **1996**, *44*, 3817−3820.
(7) Brimer, L.; Dalgaard, L. Cyanogenic glycosides and cyanohydrins in plant tissues. qualitative and quantitative determination by enzymatic post-column cleavage and electrochemical detection after separation by HPLC. *J. Chromatogr. A* **1984**, *303*, 77−88.
(8) Dicenta, F.; Martínez, P.; Berenguer, V.; Grané, N.; Martín, M. L.; Mejía, M. D. Herencia del contenido de amigdalina y del sabor de la semilla en una descendencia de almendro. Actas VIII Congreso Nacional de Ciencias Hortícolas, Murcia, 1999; pp 35−39.
(9) Nahrstedt, A. Zur cyanogenese von Prunus Avium. *Phytochemistry* **1972**, *11*, 3121−26.
(10) Baar, S. The microdetermination of cyanide: its application to the analysis of whole blood. *Analyst* **1966**, *91*, 268−272.
(11) Asmus, E.; Garschagen, H. Über Verwendung der Barbitursäure für die photometrische Bestimmnung von Cyanid und Rhodanid. *Z. Anal. Chem.* **1953**, *138*, 414−422.
(12) Cooke, R. D. An enzymatic assay for the total cyanide content of cassava (*Manihot esculenta* Crantz). *J. Sci. Food Agric.* **1978**, *29*, 345−352.
(13) Kobaisy, M.; Oomah, B. D.; Mazza, G. Determination of cyanogenic glucosides in flaxseed by barbituric acid in pyridine, pyridinpyrazolone and high performance chromatographic methods. *J. Agric. Food Chem.* **1996**, *44*, 3178−3181.
(14) Eaton, A. D., Clesceri, L. S., Greengerg A. E., Eds. *Standard methods for examination of water and wastewater*, 19th ed.; American Public Health Association: New York, 1995; 4500-CN.
(15) Arrazola-Paternina, G. Análisis de glucósidos cianogénicos en variedades de almendro: implicaciones en la mejora genética. Thesis, Universidad de Alicante, Spain, 2002.
(16) Reavy, P. F. An improved procedure for the isolation of dhurrin. *Phytochemistry* **1969**, *8* (11), 2559−2560.
(17) Chaimbault, P.; Elfakir, C.; Lafosse, M. Comparison of the retention behavior of polyethoxilated alcohols on porous graphitic carbon and polar as well as apolar bonded-silica phases. *J. Chromatogr. A* **1988**, *797*, 83−91.
(18) Stefansson, M.; Lu, B. Ion-pair of sugars at alkaline pH and pulsed electrochemical detection. *Chromatographia* **1993**, *35* (1−2), 125−131.
(19) Huynh, N. H.; Karlsson, A.; Petterson, C. Enantiomeric separation of basic drugs using N-benzyloxycarbonylglycyl-L-proline as counter ion in methanol. *J. Chromatogr. A* **1995**, *705* (2), 275−287.
(20) Creaser, C. S.; Al-Haddad, A. Fractionation of polychlorinated biphenyls, poly- chlorinated dibenzo-p-dioxins and polychlorinated dibenzofurans on porous graphitic carbon. *Anal. Chem.* **1989**, *61*, 1300−1302.
(21) Knox, J. H.; Ross, P. Carbon-based packing materials for liquid chromatography. *Adv. Chromatogr.* **1997**, *37*, 121−162.

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