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Identification of the constituent flavanoid units in sainfoin proanthocyanidins by reversed-phase high-performance liquid chromatography

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ABSTRACT

A rapid and sensitive method for the separation and identification of flavan-3-ols and their phloroglucinol adducts using reversed-phase high-performance liquid chromatography is reported. The application of the method is demonstrated for the analysis of the degradation products of sainfoin leaf proanthocyanidins. The results showed that the extension and terminal units in this sample are catechin, epicatechin, gallocatechin and epigallocatechin.

INTRODUCTION

Proanthocyanidins (condensed tannins) are a group of phenolic polymers which are widely distributed in the plant kingdom particularly in plants with a woody growth habit [1–4]. These compounds consist of flavan-3-ol (Fig. 1) units, linked together through C-4–C-6 or C-4–C-8 bonds [5]. Structural variation of proanthocyanidins ranges from dimers and trimers to more complex oligomers and polymers depending on the nature of the interflavanoid linkage, hydroxylation pattern and stereochemistry at the three chiral centers (carbons 2, 3 and 4) of the C-ring [4,6,7]. These polymers are found in numerous gymnosperms and angiosperms in large amounts [8] and have an important defensive function in many plants against micro organisms and predators [9]. In fruits and seed coats, considerable quantities of these compounds make an important contribution to the quality, colour and taste of the fruit and products derived from them [2,10,11] and play a major role in the preservation of beers [12].

Proanthocyanidins have been shown to have an important role in non-bloating forage crops because they bind with excess protein in the rumen and prevent the development of a gas trapping stable foam [13–15]. In the course of investigations aimed at the identification of proanthocyanidins in sainfoin leaves, we have examined the application of reversed-phase high-performance liquid chromatography (RP-HPLC) for the separation and identification of flavan-3-ol units in the proanthocyanidins of this species.

Degradation of proanthocyanidins is essential for the determination of their structural units [16]. Several methods have been used to analyze the degradation products of these compounds of which the most common are column chromatography [17], thin-layer chromatography (TLC), two-dimensional (2D) TLC [16,18,19] and spectroscopic methods [20–22]. However, these methods are time consuming, limited in separation power or require purified samples.

In this paper we report a rapid and sensitive method for the separation and identification of flavan-3-ols and their phloroglucinol adducts in proanthocyanidins by RP-HPLC. The application of the method is demonstrated for the analysis of degradation products of sainfoin proanthocyanidins.

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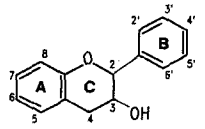
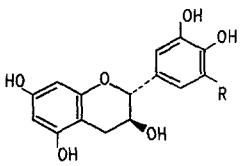
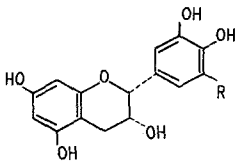


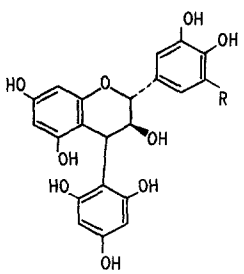
Basic structure of flavan-3-ols



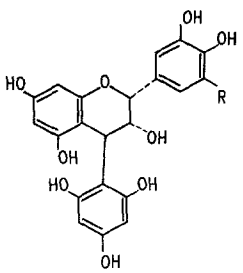
I : R = H
II : R = OH



III : R = H
IV : R = OH



V : R = H
VI : R = OH



VII : R = H
VIII : R = OH

Fig. 1. Structures of flavan-3-ols and their phloroglucinol adducts used for the HPLC analysis.

EXPERIMENTAL

Apparatus

The high-performance liquid chromatograph was a Millipore-Waters (Milford, MA, USA) equipped with a Model 600E multisolvent delivery system and a Model 994 programmable photodiode array detector. A prepacked analytical column (25 cm × 4 mm I.D.) of LiChrospher 100 RP-18 (5 μm) (E. Merck, Darmstadt, Germany) was used for all experiments. The guard column (4 mm × 4 mm I.D.) was packed with the same material.

Elution

Two solvents were used: A = 1% aqueous acetic acid; B = methanol. The elution system was: 0–30 min, 0–15% B in A (linear gradient); 30–45 min, 15–60% B in A (linear gradient); 45–50 min, 60% B in A (isocratic). The column temperature was ambient and the flow-rate was set at 1 ml min^{-1}.

Detection

The photodiode array detector was set to monitor the chromatograms at 280 nm and acquire the UV spectra between 200–400 nm.

Materials

The solvents chloroform (Fisher Scientific, Ottawa, Canada), acetone and ethyl acetate (BDH, Toronto, Canada) were of analytical grade. The methanol (BDH) used for the analysis was of HPLC grade. Sainfoin leaves, *Onobrychis viciifolia* Scop. (var. Melrose), were supplied by Agriculture Canada, Saskatoon. Phloroglucinol was obtained from Aldrich (Milwaukee, WI, USA) and recrystallized before use. (−)-Epigallocatechin was purchased from Apin Chemicals (Abingdon, UK). (−)-Epicatechin and (+)-catechin were obtained from Sigma (St. Louis, MO, USA). (+)-Gallocatechin and (+)-gallocatechin-, (−)-epigallocatechin-, (−)-epicatechin- and (+)-catechin-4-phloroglucinol adduct standards were kindly supplied by L. Y. Foo (DSIR, Petone, New Zealand). Some of these compounds contained impurities, but all consisted of one major component. These compounds were used without further purification.

Isolation and purification of sainfoin leaf proanthocyanidins

A sample of finely ground sainfoin leaves (200 g, dry weight) was extracted with 4 × 250 ml 75% aqueous acetone containing 0.1% ascorbic acid according to the method of Foo and Porter [23]. The acetone was removed under reduced pressure at ≤30°C. The aqueous solution was filtered through a plug of glasswool. The filtrate was then extracted with 3 × 300 ml chloroform. The chloroform extract was discarded and the aqueous phase was filtered through a plug of glasswool. The filtrate was extracted with 3 × 1000 ml ethyl acetate. The ethyl acetate-soluble fractions were combined and stored at 4°C for further analysis. The aqueous fraction was mixed with the same volume of methanol and chromatographed on Sephadex LH-20 column (15 cm × 4 cm I.D.). The column was washed with 50% aqueous methanol (600 ml) and proanthocyanidins were then eluted with 75% aqueous acetone

(500 ml). A brown solid (3.65 g) was obtained following the removal of acetone and freeze-drying the aqueous fraction.

Preparation of the phloroglucinol adducts of sainfoin proanthocyanidins

A sample of sainfoin proanthocyanidins (12 mg) together with 8 mg of phloroglucinol were dissolved in 100 μl 1% HCl in ethanol. The mixture was shaken vigorously and allowed to stand at room temperature for 2 h. The solvent was evaporated by a stream of dry nitrogen and the residue was dissolved in 50 μl distilled water. The latter was extracted with 2 × 100 μl ethyl acetate. The ethyl acetate extracts were combined and evaporated to dryness by a stream of dry nitrogen. The residue was dissolved in 50 μl of 70% aqueous methanol. This solution was used for injection onto the HPLC column.

Analysis of ethyl acetate-soluble fraction

The ethyl acetate-soluble fraction was evaporated to obtain a concentrated solution which was applied to a LH-20 column (30 cm × 3.5 cm I.D.). The elution was initiated with 1000 ml ethanol (five fractions, 1–5, were collected) and followed by ethanol–acetone (19:1, v/v) [22] which yielded one fraction (6). Each fraction was evaporated and the residue was dissolved in 100 μl 70% aqueous methanol and tested for flavan-3-ols and di- or trimeric proanthocyanidins by 2D-TLC and HPLC.

2D-TLC procedure

The preliminary detection of dimeric proanthocyanidins, flavan-3-ols and their phloroglucinol adducts was carried out using the standard method of two-dimensional cellulose TLC [3,16]. The chromatograms were developed in *tert.*-butanol–acetic acid–water (3:1:1, v/v/v) in the first direction and 6% acetic acid in the second direction. Compounds were visualized by spraying the chromatograms with freshly prepared 4% vanillin in methanol-conc. HCl (4:1, v/v). Red or purplish-red spots were produced after spraying and warming.

Preparation of standard samples

All standards were prepared at 1 mg ml^{-1} concentration in 70% aqueous methanol.

RESULTS AND DISCUSSION

The retention times, t_R, for some typical flavan-3-ols and their phloroglucinol adducts (Fig. 1) are given in Table I. These results demonstrate the potential of HPLC as an alternative to the other chromatographic methods for such separations reported by other workers [16–19]. Fig. 2 shows the chromatogram obtained for a mixture of these compounds. All the peaks are well resolved except for (−)-epicatechin-4-phloroglucinol (**VII**) and (+)-catechin-4-phloroglucinol (**V**) which form a critical pair. However, under the conditions reported here a sufficient separation of these two peaks was

TABLE I

RETENTION TIMES OF SOME FLAVAN-3-OLS AND THEIR PHLOROGLUCINOL ADDUCTS

HPLC conditions: column, LiChrospher 100 RP-18 (25 cm × 4 mm, I.D., 5 μm); flow-rate, 1 ml min^{-1}; column temperature, ambient; detection, 280 nm.

No.	Compound	OH Position	Absolute configuration	t_R (min)
I	(+)-Catechin	3,5,7,3′,4′	2*R*:3*S*	39.56
II	(+)-Gallocatechin	3,5,7,3′,4′,5′	2*R*:3*S*	23.06
III	(−)-Epicatechin	3,5,7,3′,4′	2*R*:3*R*	44.68
IV	(−)-Epigallocatechin	3,5,7,3′,4′,5′	2*R*:3*R*	38.49
V	(+)-Catechin-4-phloroglucinol	3,5,7,3′,4′	2*R*:3*S*	26.47
VI	(+)-Gallocatechin-4-phloroglucinol	3,5,7,3′,4′,5′	2*R*:3*S*	13.61
VII	(−)-Epicatechin-4-phloroglucinol	3,5,7,3′,4′	2*R*:3*R*	25.83
VIII	(−)-Epigallocatechin-4-phloroglucinol	3,5,7,3′,4′,5′	2*R*:3*R*	16.74

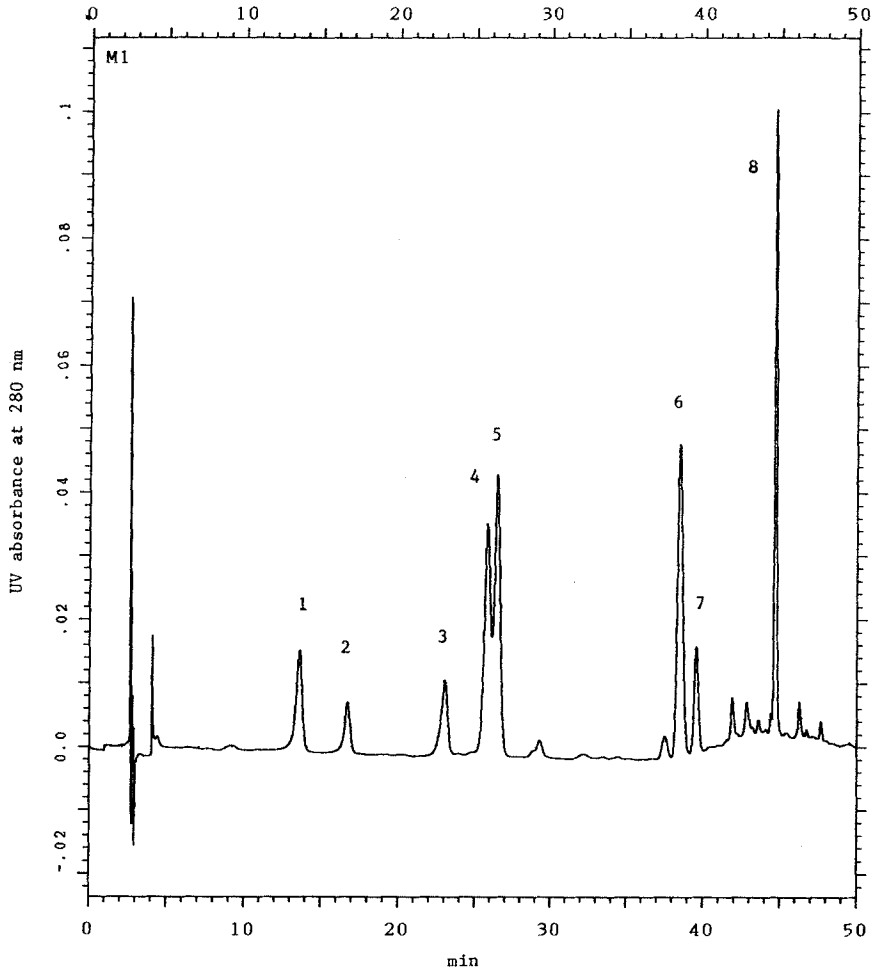


Fig. 2. HPLC of a standard mixture of flavan-3-ols and their phloroglucinol adducts using a LiChrospher 100 RP-18 column (25 cm × 4 mm I.D., 5 μm) at a flow-rate of 1 ml min^{-1}. For the elution system see Experimental. Peaks: 1 = (+)-gallocatechin-4-phloroglucinol; 2 = (−)-epigallocatechin-4-phloroglucinol; 3 = (+)-gallocatechin; 4 = (−)-epicatechin-4-phloroglucinol; 5 = (+)-catechin-4-phloroglucinol; 6 = (−)-epigallocatechin; 7 = (+)-catechin; 8 = (−)-epicatechin.

achieved (Fig. 2). Since the structures of these two compounds (**V** and **VII**) differ only in the stereochemistry at C-3, a better separation may be obtained using a chiral HPLC column [24]. As a general rule, the substitution pattern of the B-ring is an important factor in the elution order of these compounds.

The retention times of the compounds with three hydroxyl groups on the B-ring (*i.e.* compounds with pyrogallol group) are shorter than those of the corresponding compounds with two hydroxyl groups (*i.e.* compounds with cathechol group). Thus the elution order for flavan-3-ols is: (+)-gallocatechin (**II**) < (−)-epigallocatechin (**IV**) < (+)-catechin (**I**) < (−)-epicatechin (**III**). This is true in the case of phloroglucinol adducts as well, since (+)-gallocatechin-4-phloroglucinol (**VI**, 13.61 min) and (−)-epigallocatechin-4-phloroglucinol (**VIII**, 16.74 min) are eluted before (−)-epicatechin-4-phloroglucinol (**VII**, 25.83 min) and (+)-catechin-4-phloroglucinol (**V**, 26.47 min). As expected, all the phloroglucinol adducts (compounds **V**–**VIII**) are eluted faster than their corresponding flavan-3-ols (compound **I**–**IV**). This can be attributed to the polarity of these compounds which is greater than the corresponding flavan-3-ols due to the substitution of a phlorogluci-

nol group at the C-4 positions resulting in the formation of stronger hydrogen bonds with the mobile phase. In addition to the substitution pattern of the B-ring and the polarity of the compounds, the stereochemistry at the C-3 (*i.e.* the stereochemical position of the OH group at this position) also influences the elution order. For example, (+)-catechin (**I**) with 2*R*:3*S* configuration has a shorter retention time than (−)-epicatechin (**III**) with 2*R*:3*R* configuration (39.56 min and 44.68 min, respectively). In the same way, (+)-gallocatechin, **II** (2*R*:3*S*) eluted before (−)-epigallocatechin, **IV** (2*R*:3*R*) at the retention times of 23.06 min and 38.49 min, respectively.

Identification of the constituent flavanoid units in sainfoin proanthocyanidins was carried out by degradation of these compounds using acid hydrolysis in the presence of phloroglucinol. Degradation of these compounds with acids in the presence of various nucleophiles is a well known method since the stereochemistry at C-2 and C-3 positions is preserved [16]. Although acid-catalyzed thiolysis has been used by many workers [22,25,26], it requires a long procedure and has an offensive odour. However, the use of phloroglucinol as the nucleophile is more convenient and offers a better separation of the degradation products when using different chromatographic systems [16]. The results obtained

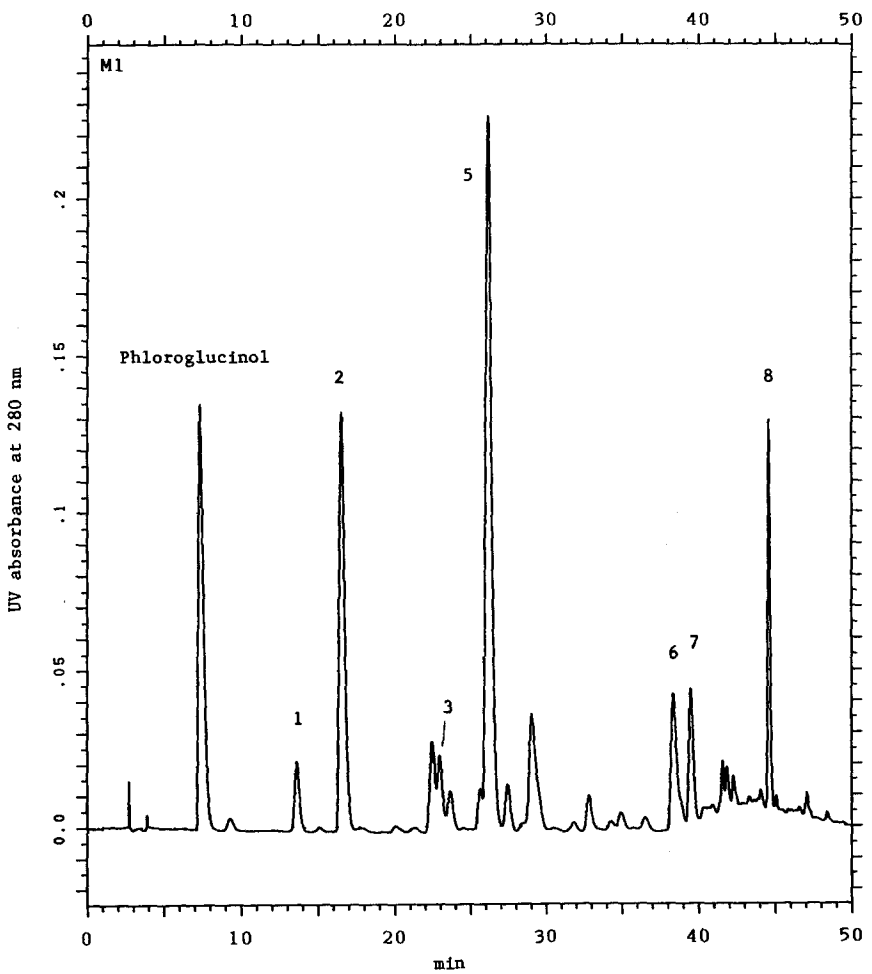
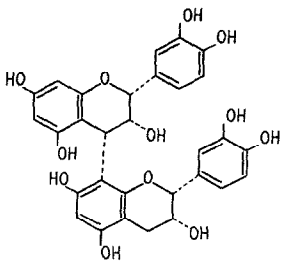


Fig. 3. HPLC of the degradation products of sainfoin leaf proanthocyanidins. HPLC conditions and peak identification as for Fig. 2.

from the time course studies of sainfoin proanthocyanidin hydrolysis in present work indicated that a total of 2 h time was sufficient for the completion of hydrolysis.

The flavan-3-ols and their phloroglucinol adducts discussed below were identified by the comparison of their retention times and in some cases UV spectra with those of authentic standards. Analysis of the degradation products of sainfoin leaf proanthocyanidins by RP-HPLC, showed that (+)-catechin (**I**), (+)-gallocatechin (**II**), (−)-epicatechin (**III**), (−)-epigallocatechin (**IV**) and (+)-catechin-, (+)-gallocatechin- and (−)-epigallocatechin-4-phloroglucinol adducts (compounds **V**, **VI** and **VIII**, respectively) are present in sainfoin sample (Fig. 3). The extension and terminal units in sainfoin proanthocyanidins, therefore, are catechin, gallocatechin, and epigallocatechin, while epicatechin appears to be terminal unit only.

The ethyl acetate-soluble fraction of sainfoin leaves (see Experimental) was also tested for the detection of any low molecular weight proanthocyanidins (dimers and trimers) and/or flavan-3-ols, if present. This sample was applied to a LH-20 column and eluted with ethanol and ethanol–acetone (19:1, v/v) which yielded six fractions. All the fractions were monitored by 2D-TLC [3,16] of which fractions 5 and 6 showed four and two purple spots, respectively indicating the presence of flavan-3-ols or low-molecular-weight proanthocyanidins. Application of the HPLC method proposed in this paper, showed that the major component in fraction 5 was (−)-epicatechin (**III**) while traces of (+)-catechin (**I**), (+)-gallocatechin (**II**) and (−)-epigallocatechin (**IV**) were also detected. Two purple spots in the 2D-TLC of fraction 6 were tentatively identified as (−)-epigallocatechin (**IV**) and a dimer by comparison with data from literature [3,16]. Isolation and purification of these two compounds by preparative HPLC and further analysis, confirmed the result obtained from 2D-TLC that one of the compounds is (−)-epigallocatechin (**IV**). Degradation of the dimer followed by HPLC analysis showed only two peaks in the chromatogram. Comparison of the retention times of these peaks with those of standards (Table I) showed that they are (−)-epicatechin (**III**) and its phloroglucinol adduct (**VII**). These results indicate that the dimer could be (−)-epicatechin-(4β-8)-epicatechin (**IX**) as the extension and terminal units are both epicatechin.



(IX)

RP-HPLC analysis of the degradation products of proanthocyanidins offers an attractive method for the rapid and sensitive separation and identification of structural units in these compounds. The adoption of this technique provides greater resolution, better sensitivity than other chromatographic methods and requires only a small amount of the proanthocyanidins. This method is currently being used for the structural and biosynthetic studies of sainfoin leaf proanthocyanidins in our laboratory.

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