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Isolation and determination of flavonol glycosides from *Epilobium* species

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ABSTRACT

Centrifugal partition chromatography has been applied to the separation of flavonol glycosides from *Epilobium parviflorum* (Onagraceae). Using these glycosides, a method is described for their determination in different species of *Epilobium* by high-performance liquid chromatography on an octadecylsilyl column.

INTRODUCTION

The genus *Epilobium* (family Onagraceae) consists of over 200 species, sixteen of which are found in Switzerland and the most common being *E. angustifolium*. The commercially available plant drug *Epilobii herba* contains the dried aerial parts of several species, most notably *E. parviflorum*, *E. montanum* and *E. roseum*. Various members of the genus *Epilobium*, in particular *E. parviflorum*, have been used in folk medicine for the treatment of adenoma and inflammation of the prostate [1,2]. The constituents of *Epilobium* are not well known, but the presence of sterols [3], triterpenes and flavonoids (flavonol aglycones and glycosides) [4] has been reported. The active principles have not yet, however, been identified and it has been necessary to select suitable constituents for standardisation purposes. As the flavonoid glycosides occur in relatively large amounts, they have been chosen as markers.

The first part of this investigation involved the isolation of the major flavonoid glycosides quercitrin (1), myricitrin (2) and isomyricitrin (3) from *E. parviflorum*, using centrifugal partition chromatography (CPC) as the central separation step. This is a recently developed liquid–liquid technique which allows rapid separations without the problems of irreversible adsorption and decomposition which frequently occur in chromatography on solid supports [5,6]. CPC has multiple applications, especially in the area of polar natural products [7].

The determination of quercitrin, myricitrin and isomyricitrin in several different species of *Epilobium* was then undertaken. Finally, the extracts were hydrolysed to determine the principal aglycones formed.

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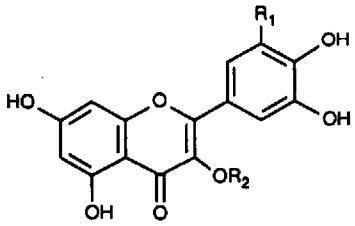

1	R_1 = H	R_2 = Rha	(quercitrin)
2	R_1 = OH	R_2 = Rha	(myricitrin)
3	R_1 = OH	R_2 = Glc	(isomyricitrin)
4	R_1 = H	R_2 = Glc	(isoquercitrin)
5	R_1 = H	R_2 = H	(quercetin)
6	R_1 = OH	R_2 = H	(myricetin)

EXPERIMENTAL

Apparatus

CPC was performed at *ca.* 20°C with an Ito multi-layer coil separator–extractor (P.C., Potomac, MD, USA), equipped with a 2.6 mm I.D. coil (volume 360 ml), sample loop and valve to allow switching of the solvent to the "head" or "tail" ends of the coil. Solvent delivery was by two Waters Assoc. 6000A high-performance liquid chromatography (HPLC) pumps [8]. The instrument was filled with 80% upper phase and 20% lower phase, and connected to a Büchi 683 detector (254 nm) (Büchi, Flawil, Switzerland), a Model 600 chart recorder (W + W Scientific, Basle, Switzerland) and an Ultrorac II fraction collector (Pharmacia, Bromma, Sweden).

HPLC–UV analyses were carried out on a system consisting of a Spectra-Physics 8700 pump (San Jose, CA, USA), a Rheodyne injector, a Hewlett-Packard (HP) 1040A photodiode array detector (Palo Alto, CA, USA), an HP-85 computer and an HP 7470A plotter. Quantitative analyses were performed with a Spectra-Physics 8800 pump connected to a Pharmacia 2151 variable-wavelength monitor and a Pharmacia 2221 integrator.

The chromatographic separations were run on Waters 4 μm NovaPak C_{18} (15 cm × 3.9 mm I.D.) and 10 μm μBondapak C_{18} (30 cm × 3.9 mm I.D.) columns and the samples were filtered over HV 0.45 μm units (Millipore, Bedford, MA, USA).

Plant material

Rosebay willowherb (*E. angustifolium*) was purchased from Siegfried (Zofingen, Switzerland), while the other species of *Epilobium* were collected at the Domaine expérimental de Bruson (Station fédérale de recherche agronomique, CH-1934 Bruson, Valais, Switzerland) in July 1989.

Isolation of flavonoid glycosides

The aerial parts (200 g) of *E. parviflorum* were extracted first with dichloromethane (3 × 2000 ml) and then with methanol (3 × 2000 ml). A part (2 × 2 g) of the

methanol extract (16.3 g) was chromatographed by CPC, using the solvent system chloroform–methanol–water (7:13:8, v/v), with the lower phase as the mobile phase. Four major fractions (I–IV) were obtained. Gel permeation chromatography (GPC) of fraction I on Sephadex LH-20 (methanol) yielded quercitrin (1) (25 mg), whereas GPC of fraction II under the same conditions gave myricitrin (2) (35 mg), isomyricitrin (3) (18 mg) and gallic acid (26 mg). The structures of 1–3 were confirmed by UV, mass spectrometry, ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy, acidic hydrolysis and comparison with authentic samples.

Chromatographic conditions

The separations of glycosides were performed on a NovaPak column with a linear gradient of acetonitrile in water (10:90 to 25:75), each containing 2% orthophosphoric acid, over 30 min. The flow-rate was 1 ml/min and detection was at 350 nm. For the aglycones, a μBondapak column and a linear gradient of acetonitrile–water (20:80 to 60:40) over 20 min were used.

Quantitative analyses

The aerial parts (2.0 g) of each *Epilobium* species were extracted with 25% aqueous ethanol (200 ml) at room temperature for 24 h. After filtration, the solvent was removed by evaporation under reduced pressure and lyophilisation. A portion of the residue (10 mg) was taken up in methanol (1 ml).

Quantitative determinations were carried out with the flavonol aglycone myricetin as an internal standard. A 1 ml volume of a 1 mg/ml solution in methanol was added to 2 ml of a 10 mg/ml solution of *Epilobium* extract and 10 μl of the resulting solution was injected onto the HPLC column. A solution containing 0.25 mg/ml of the standard and each of the three flavonol glycosides 1–3 was used to calculate the correction factors. To obtain the standard correction factor (SCF) for the three glycosides, different volumes of the mixture were each injected three times and the SCF was calculated as follows:

$$\mathrm{SCF} = \frac{A(\mathrm{L})\ W(\mathrm{St})}{A(\mathrm{St})\ W(\mathrm{L})}$$

where $A(\mathrm{L})$ = peak area of flavonol glycoside, $A(\mathrm{St})$ = peak area of myricetin standard, $W(\mathrm{L})$ = weight of flavonol glycoside and $W(\mathrm{St})$ = weight of myricetin standard. The correction factors calculated were: quercitrin = 1.74, myricitrin = 1.67 and isomyricitrin = 1.73.

The linearity of the relationship between peak area and amount injected (over the range used for the determination) was checked by constructing calibration graphs for each of the flavonol glycosides.

The flavonol glycosides 1–3 in *Epilobium* extracts were determined by injecting samples with myricetin (prepared as described above) and calculating their content from the standard correction factors. HPLC runs were performed in triplicate to obtain the average results.

Hydrolyses

A portion (20 mg) of the 25% ethanolic plant extract was treated with 2 *M*

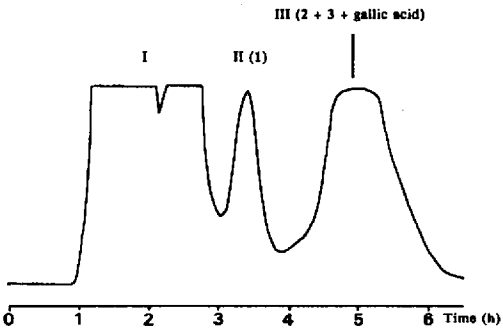

Fig. 1. CPC of a methanol extract of *E. parviflorum* (Onagraceae) aerial parts on a multi-layer coil separator–extractor. Solvent, chloroform–methanol–water (7:13:8) (mobile phase = lower phase; 80% stationary phase in coil); flow-rate, 3.5 ml/min; 700 rpm; detection, 254 nm; sample, 2 g.

hydrochloric acid (50 ml) at 70°C for 1 h. After cooling, the acidic solution was extracted with ethyl acetate (3 × 50 ml) and the organic layer was washed with water (2 × 50 ml). After evaporation, the residue was dissolved in methanol (1 ml) and 10 μl of this solution was injected for HPLC analysis.

RESULTS AND DISCUSSION

The initial isolation step for obtaining flavonol glycosides from the aerial parts of *E. parviflorum* was liquid–liquid partition chromatography on the Ito multi-layer coil separator–extractor. Using a chloroform–methanol–water solvent system, charges of 2 g could be loaded onto the instrument (Fig. 1). As the lower phase was used as the mobile phase, lipophilic compounds eluted at the beginning of the separation (band I). Of the glycosides, the first to elute was quercitrin (1), contained in band II (60 mg). This only needed a subsequent GPC step for final purification. Three substances eluted together under peak III (130 mg). This mixture of myricitrin (2), isomyricitrin (3) and gallic acid was separated by Sephadex LH-20 GPC. The three glycosides obtained in this way were used as reference compounds. The remainder of the extract injected into the chromatograph was eluted by running the upper phase of the two-phase system as the mobile phase and changing the direction of flow.

Quantitative analyses of the flavonol glycosides were performed with myricetin (6) (the aglycone of 2 and 3) as internal standard, as this compound elutes just after the glycosides and does not interfere with the peaks important for the determinations. A chromatogram of the 25% ethanolic extract of the aerial parts of *E. parviflorum* is shown in Fig. 2, with detection at 350 nm. Myricitrin (2) is the major constituent, while quercitrin (1) and isomyricitrin (3) occur in smaller amounts. Trace amounts of other polyphenolic glycosides are present and chlorogenic acid elutes at 3.8 min (identified by co-elution and comparison of the UV spectra with an authentic sample). The UV spectra of the flavonol glycosides were obtained by the use of a diode-array detector.

A similar chromatogram for the ethanolic extract of the aerial parts of *E. roseum* is shown in Fig. 3. Again, myricitrin (2) is the major component. Chlorogenic acid (retention time 3.9 min) is present in higher concentrations than for *E. parviflorum*. The

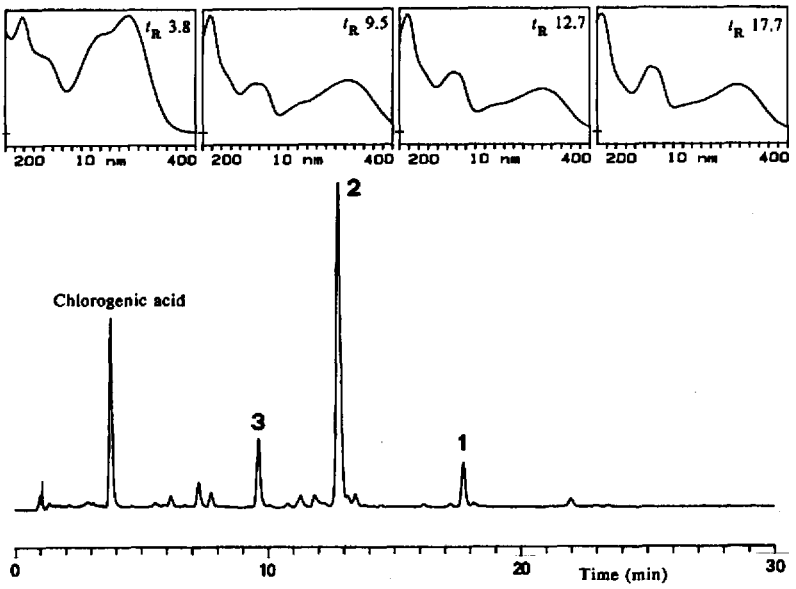

Fig. 2. HPLC separation of a 25% ethanol extract of the aerial parts of *E. parviflorum*. (1) Quercitrin, (2) myricitrin, (3) isomyricitrin. Column, NovaPak C_{18} (15 cm × 3.9 mm I.D.); eluent, 10% acetonitrile (containing 2% orthophosphoric acid) increasing to 25% acetonitrile over 30 min; flow-rate, 1 ml/min; detection, 350 nm.

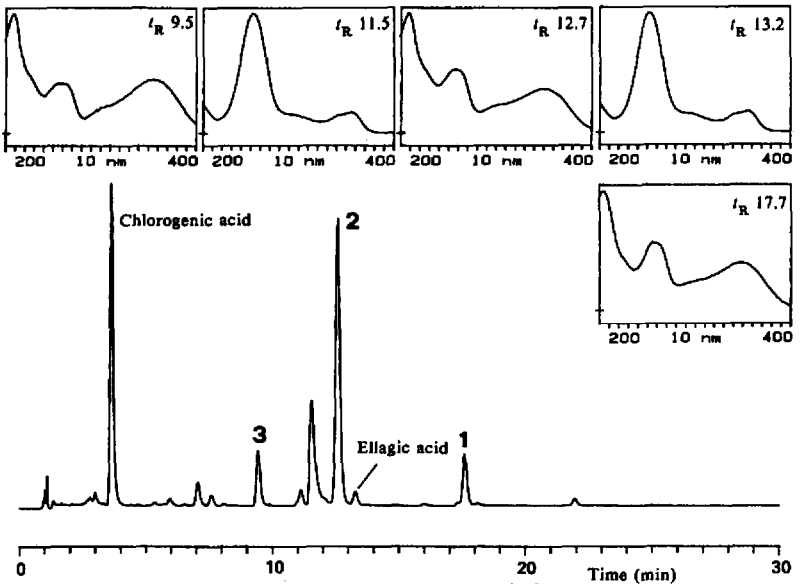

Fig. 3. HPLC separation of a 25% ethanol extract of the aerial parts of *E. roseum*. Conditions and peaks as in Fig. 2.

TABLE I

QUANTITATIVE DETERMINATION OF FLAVONOL GLYCOSIDES IN 25% ETHANOLIC EXTRACTS OF AERIAL PARTS OF EUROPEAN *EPILOBIUM* SPECIES

Species	Glycosides (%)		
	Quercitrin (1)	Myricitrin (2)	Isomyricitrin (3)
Epilobium parviflorum B1	0.24	2.13	1.31
Epilobium roseum C2	0.23	2.26	0.23
Epilobium montanum B1	0.23	2.08	1.23
Epilobium hirsutum B1	0.22	1.50	0.55
Epilobium hybridum B1	0.23	1.94	1.00
Epilobium dodonaei C1	0.22	1.49	0.92
Epilobium tetragonum B1	0.23	1.36	1.37
Epilobium alpinum C1	0.26	4.48	0.55

peak at 13.2 min was identified as ellagic acid by comparison with an authentic sample, whereas the peak eluting at 11.5 min has a UV spectrum very similar to that of ellagic acid and is not of a flavonoid nature.

The amounts of the three marker flavonol glycosides 1–3 in the aerial parts (ethanol extract) of eight different species of *Epilobium* collected in Valais, Switzerland, are shown in Table I. All contain differing amounts of the three glycosides. Except for *E. tetragonum*, myricitrin is always present in the largest amount. The highest percentage of myricitrin is found in *E. alpinum*. Myricitrin and isomyricitrin occur in equal amounts in *E. tetragonum* but, as in all the other species, the amount of quercitrin is the smallest of the three glycosides.

Analysis of the commonly found *E. angustifolium* gives a completely different flavonoid pattern from the other species. Instead of having myricitrin as the main

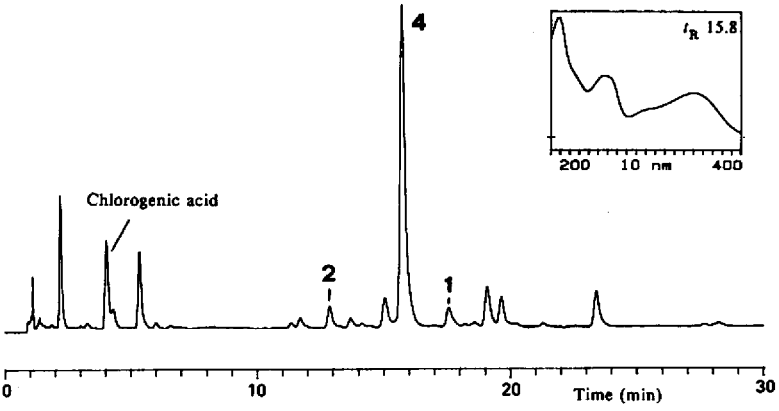

Fig. 4. HPLC separation of a 25% ethanol extract of the aerial parts of *E. angustifolium*. (4) Isoquercitrin. Conditions as in Fig. 2.

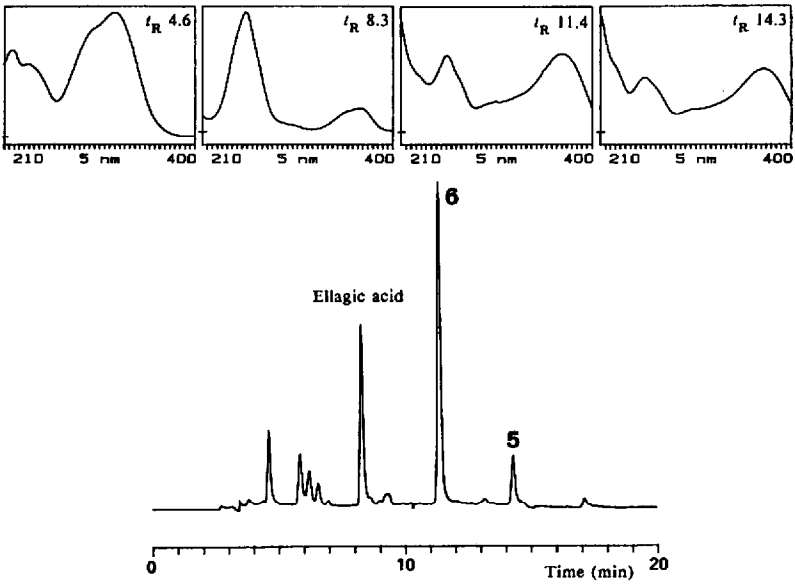

Fig. 5. Analytical HPLC of the acid hydrolysate from a 25% ethanolic extract of *E. parviflorum* aerial parts. 5 = Quercetin; 6 = myricetin. Column, μBondapak C_{18} (30 cm × 3.9 mm I.D.); eluent, 20% acetonitrile (containing 2% orthophosphoric acid) increasing to 60% acetonitrile over 20 min; flow-rate, 1 ml/min; detection, 350 nm.

constituent, isoquercitrin (4) (retention time 15.8 min) was identified as the most important flavonoid in the ethanolic extract (Fig. 4). A small amount of chlorogenic acid was also present (retention time, t_R 3.9 min). The analytical HPLC method described here thus provides a useful means of distinguishing the large-flowered *E. angustifolium* from the smaller-flowered species shown in Table I.

Acidic hydrolysis of the *Epilobium* extracts gave the corresponding aglycones, quercetin (5) and myricetin (6), of the glycosides 1–3. This can be seen in the HPLC chromatogram of the *E. parviflorum* hydrolysate (Fig. 5). Myricetin (6) is the predominant aglycone, with smaller amounts of quercetin (5). A trace of kaempferol elutes with a retention time of 17.2 min. The peak at t_R 4.6 min has a UV spectrum (diode-array detector) very similar to that of chlorogenic acid, while the slower-running peak (t_R 8.3 min) has a UV spectrum characteristic of phenolic acids; this was confirmed as ellagic acid by comparison with an authentic sample.

CONCLUSIONS

CPC is a very rapid and efficient technique for the fractionation of crude extracts. In the application illustrated here, the initial purification of a relatively large (2 g) amount of polar phenolic glycosides was possible, without encountering the problems of irreversible adsorption frequently encountered when using chromatography with solid supports. For the final purification of the glycosides, a single GPC step was necessary.

The three flavonol glycosides isolated are useful markers for the standardisation

of different batches of *Epilobium* plant material as they are present in all the small-flowered species analysed. Their determination is straightforward and involves a simple extraction step with ethanol. In all the samples investigated, no interference from other compounds was experienced. Thus, although the active principles in *Epilobium* are not yet known, a reliable method for the quality control of the crude plant drug now exists. This should be of considerable use when comparing different batches of *Epilobium* species from large-scale cultivation.

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