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Note

Determination of isoflavones from *Ononis spinosa* L. extracts by high-performance liquid chromatography with ultraviolet diode-array detection

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The root extracts of *Ononis spinosa* L. are known[1] to be diuretically active. In a previous paper[2] we reported the high-performance liquid chromatographic (HPLC) determination of flavonoids from *Ononis spinosa* L. roots. Isolation of these components was carried out by extraction of the tissue with 50% ethanol at 70°C. Chromatographic separation was then performed on a μBondapak C_{18} column by gradient elution with acetonitrile–water (pH 2.6). However, isocratic conditions are preferred for quantitative analysis because of the greater reproducibility, especially when routine determinations are required.

In this respect, it has been recently been shown that eluents containing C_3 alcohols and ethers have great potential for the isocratic HPLC determination of flavonoids in different extracts, such as *Passiflora incarnata L.*[3], *Crataegus monogyna*[3], *Matricaria chamomilla*[4], *Ginkgo biloba*[5] and *Betulae folium*[6]. This paper describes a similar approach for the determination of genistein, formononetin and biochanin A in *Ononis spinosa* L. extracts.

Isolation of the isoflavones is carried out by percolation of the extract through a Sep-Pak C_{18} cartridge, washing with water followed by water–acetonitrile (75:25) and selective elution of the retained isoflavones with methanol. Determination of the isolated isoflavones is carried out by HPLC on a C_8 Aquapore RP 300 column and UV diode-array detection (DAD). Elution is isocratic using 2-propanol–tetrahydrofuran–water (28:2:70).

EXPERIMENTAL

Materials

Authentic samples of genistein (ICN Pharmaceuticals, Plainview, NY, U.S.A.), formononetin (Extrasynthese, Genay, France) and biochanin A (Aldrich Europe,

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Beerse, Belgium) were purchased as standards. *Ononis spinosa* root extracts were obtained from different commercial sources.

2-Propanol and tetrahydrofuran were of HPLC grade (Baker, Deventer, The Netherlands). Sep-Pak C_{18} cartridges (Waters Assoc., Milford, MA, U.S.A.) were used for sample preparation.

Apparatus

The HPLC analysis was carried out on a Waters Assoc. liquid chromatograph equipped with a Model U6K universal injector and a Model 510 pump connected to a Model HP 1040A photodiode-array detector (Hewlett-Packard, Waldbronn, F.R.G.). The column used was a C_8 Aquapore RP 300 (7 μm spherical) (250 × 4.6 mm I.D.) (Brownlee Labs., Santa Clara, CA, U.S.A.). The mobile phase was 2-propanol–tetrahydrofuran–water (28:2:70, v/v/v) at a flow-rate of 1 ml/min.

The signals were acquired (all with 4 nm band width) at 254, 280 and 310 nm. The acquisition of UV spectra was automatic at the apex, both inflection points and the base of all peaks (200–400 nm, 2-nm steps).

Purity assay of the chromatographic peaks

The UV spectra acquired for each peak, after subtraction of the corresponding UV base spectrum, were computer normalized and the plots were superimposed. Peaks were considered as chromatographically pure when there was exact coincidence among their corresponding UV spectra.

Sample preparation

A Sep-Pak C_{18} cartridge was activated by passing 3 ml of methanol followed by 5 ml of water. An 1-ml volume of *Ononis spinosa* L. hydro–alcoholic extract was diluted with 1 ml of water, percolated through the cartridge and washed with 3 ml of water and 5 ml of water–acetonitrile (3:1). The isoflavones were then eluted with methanol (4 ml). The solvent was evaporated to dryness and the residue was dissolved in methanol (1 ml).

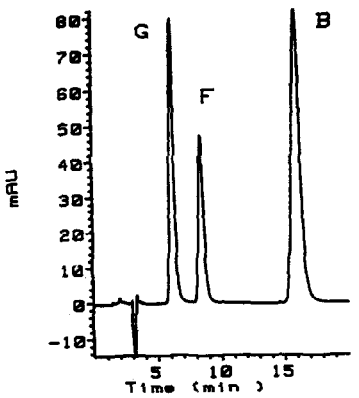
Calibration graphs

Genistein, formononetin and biochanin A were dissolved in methanol at concentrations of 1 mg/ml. These stock solutions were diluted with the eluent to obtain reference solutions containing 0.01–0.2 μg in 10 μl.

RESULTS AND DISCUSSION

The chromatogram in Fig. 1 shows a typical run with a standard mixture of genistein, formononetin and biochanin A. As indicated in Fig. 2, adequate separation of the isoflavones in *Ononis spinosa* L. extracts can be achieved in less than 18 min.

The UV spectra of genistein (G), formononetin (F) and biochanin A (B) are presented in Fig. 3. When the acquired UV spectra were computer normalized, plotted and superimposed, exact coincidence curves were obtained (match factor >990). Consequently, the peaks were assumed to be pure. The first chromatographic runs were carried out using 2-propanol–water (30:70) as eluent, and sharp peaks were obtained. However, from the DAD analysis it was clear that the formononetin peak



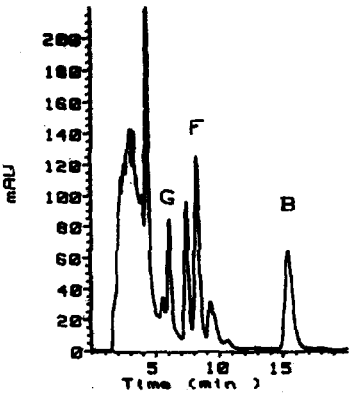


Fig. 1. Chromatogram of genistein (G, 60 ng), formononetin (F, 30 ng) and biochanin A (B, 80 ng) standards. Chromatographic conditions: column, C_8 Aquapore RP 300; eluent, 2-propanol–tetrahydrofuran–water (28:2:70); flow-rate, 1.0 ml/min; UV detection at 254 nm.

Fig. 2. Typical chromatogram of an *Ononis spinosa* L. extract. Chromatographic conditions as in Fig. 1.

was not pure. Addition of 2% of tetrahydrofuran to the 2-propanol phase allowed formononetin to be separated from later eluting components (the broad peak at 9.5 min). Further exact coincidence between the UV spectra of the peaks and those of their corresponding isoflavone standards was found, thus confirming their previous identification through the retention times.

Rectilinear responses between peak area and amounts injected were obtained from three replicate injection of genistein, formononetin and biochanin A standard solutions in the range 10–200 ng, as indicated by the following equations:

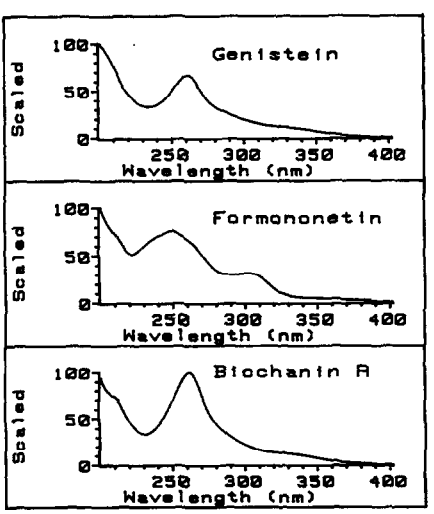


Fig. 3. UV spectra of genistein (G), formononetin (F) and biochanin A (B).

TABLE I

CONTENTS OF GENISTEIN, FORMONONETIN AND BIOCHANIN A IN THREE COMMERCIAL *ONONIS SPINOSA* EXTRACTS

Extract	*Compound (mg per 100 g)*		
	Genistein	*Formononetin*	*Biochanin A*
I	1.7	3.2	0.08
II	2.1	4.7	0.21
III	3.8	5.9	0.30

$$y = 10.4x + 0.53 \quad r = 0.998 \text{ (genistein)}$$
$$y = 26.4x + 2.31 \quad r = 0.996 \text{ (formononetin)}$$
$$y = 12.1x + 1.03 \quad r = 1.000 \text{ (biochanin A)},$$

where y represents the peak area and x the amount injected (ng).

Tests on standards gave recoveries of 98 $\pm$ 1.5% and, with a certain amount of standard added to the extract, 96.4 $\pm$ 4.2%. Intra-day relative standard deviations were determined by assaying the samples four times on the same days and were 2.9%, 3.0% and 3.4% for genistein, formononetin and biochanin A, respectively. Inter-day relative standard deviations were obtained by analysing the samples daily for a week and were 2.6%, 2.9% and 3.2% for genistein, formononetin and biochanin A, respectively.

Analysis of commercial extracts was performed by external standardization, with a good relative standard deviation of 3.2% (n = 5), and the results are reported in Table I.

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