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Note

High-performance liquid chromatographic analysis of flavonoids from *Ononis spinosa* L.

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Onosis spinosa L. is a leguminous plant widely distributed and its root extracts are diuretically active[1]. The constituents of the roots are terpenes (*trans*-anethol[2] and α-onocerin[3]), sterols (β-sitosterol) and flavonoids[4]. The content of terpenes and sterols has been determined by gas chromatography; the main components are *trans*-anethol, α-onocerin and β-sitosterol. Thin-layer and paper chromatography were recently utilized to isolate and identify flavonoid derivatives[5] from *O. spinosa*. However, for the separation and quantification of complex natural mixtures of flavonoids, high-performance liquid chromatography (HPLC) is the method of choice, as indicated by the numerous recent papers[6,7].

Extending our study[8] on HPLC analysis of medicinal plant extracts, we report in this note the HPLC determination of rutin, kaempferol, genistein, formononetin and biochanin A in *O. spinosa* roots.

EXPERIMENTAL

Materials

Authentic samples of rutin, kaempferol (Fluka, Buchs, Switzerland), genistein (ICN Pharmaceuticals, Plainview, NY, U.S.A.) and biochanin A (Aldrich-Europe, Beerse, Belgium) were obtained as standard. Formononetin was isolated according to Bradbury[9]. Benzyl-4-hydroxybenzoate (internal standard) was purchased from Formenti (Milan, Italy). *O. spinosa* roots were obtained from Brisighello (Padova, Italy), Indena (Milan, Italy), Ulrich (Torino, Italy) and Galke (Gittelde, F.R.G.). Acetonitrile was of HPLC grade (Farmitalia-Carlo Erba, Milan, Italy); deionized water was filtered through a 0.45-μm Millipore filter.

Apparatus

HPLC was performed on a Waters (Milford, MA, U.S.A.) liquid chromatograph equipped with two Model 6000 A pumps, a Model 680 automated gradient controller, a Model U6K universal injector, a Model lambda-max 480 ultraviolet

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detector and a Model 730 data module. The column was a Waters μBondapak C_{18} (30 cm × 3.9 mm I.D.); a pre-column (Waters Part no. 84550), packed with Bondapak/Corasil (37–50), was used to protect the column.

Elution

Two eluents were used: A, water (pH 2.6 with 10% phosphoric acid); B, acetonitrile. The elution profile was: 0–9 min, 72% A, 28% B, flow-rate 1.5 ml/min (isocratic); 9–10 min, 72% A, 28% B, flow-rate 1.5–2.0 ml/min (linear gradient); 10–25 min, 72–30% A, 28–70% B, flow-rate 2.0 ml/min (linear gradient).

Peaks were monitored at 263 nm and the range setting was fixed at 0.02 a.u.f.s.

Solutions

Rutin (360 μg/ml), genistein (90 μg/ml), kaempferol (500 μg/ml), formononetin

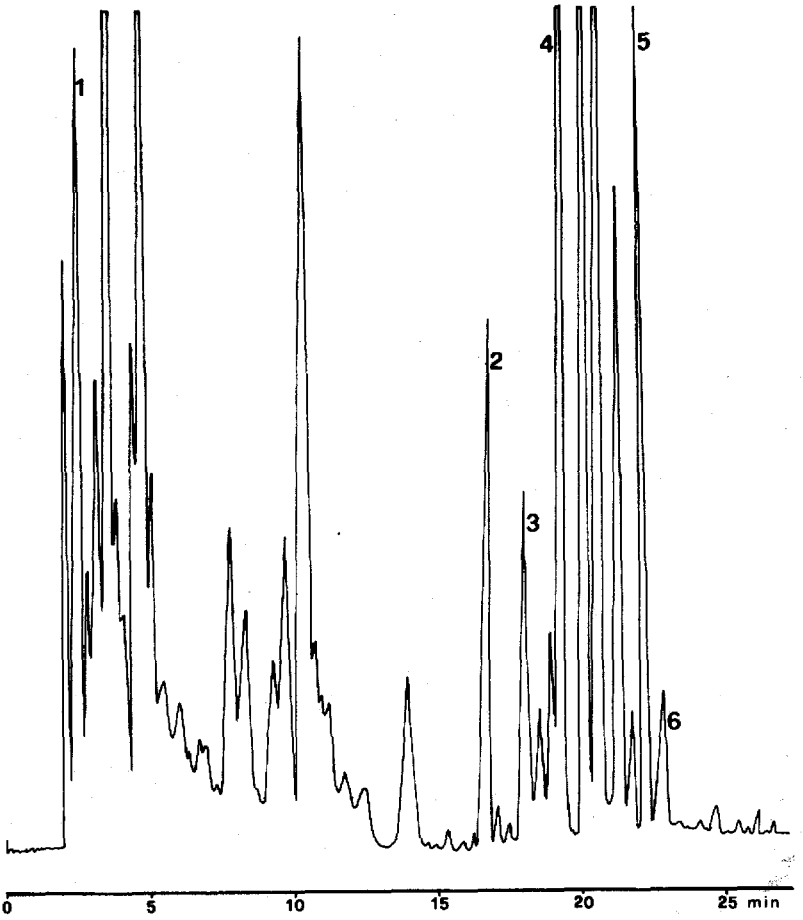

Fig. 1. High-performance liquid chromatogram of *O. spinosa* extracts. See text for eluents and elution profile. Column, μBondapak C_{18}; detector, UV 263 nm (0.02 a.u.f.s.). Peaks: 1 = rutin; 2 = genistein; 3 = kaempferol; 4 = formononetin; 5 = benzyl-4-hydroxybenzoate (internal standard); 6 = biochanin A.

(90 μg/ml) and biochanin A (66 μg/ml) were dissolved in 40% acetonitrile; benzyl-4-hydroxybenzoate (internal standard) was dissolved in the same eluent (0.5 mg/ml).

Calibration graphs

Genistein, formononetin and biochanin A (5–20 μl) were added to the internal standard (10 μl) and diluted with 40% acetonitrile to 150 μl; replicate injections of 5 μl were made for each mixture.

Sample preparation

The extracts of *O. spinosa* were prepared as follows: the tissue (1 g) was finely ground and extracted with 50% ethanol (60 ml) for 1 h at 70°C, filtered and evaporated to dryness. The residue was dissolved in 10 ml of 40% acetonitrile containing the internal standard (0.33 mg/ml). A 10-μl aliquot of each solution was injected.

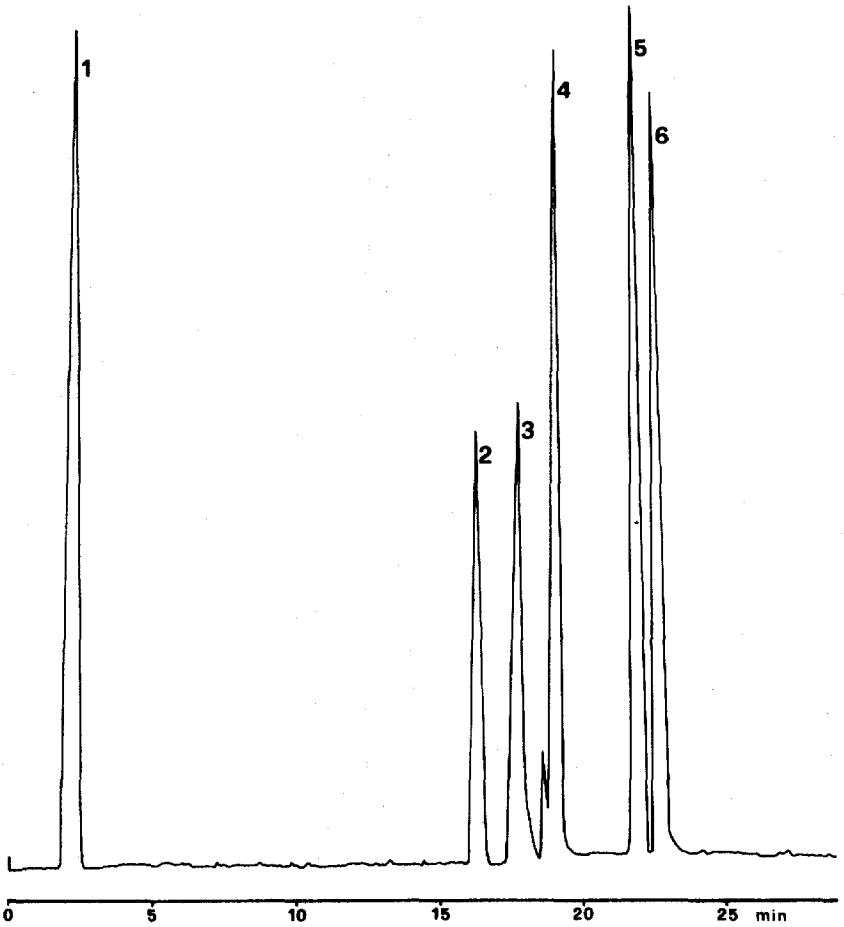

Fig. 2. High-performance liquid chromatogram of rutin (1), 180 ng; genistein (2), 15 ng; kaempferol (3), 125 ng; formononetin (4), 45 ng; benzyl-4-hydroxybenzoate (5), (internal standard); biochanin A (6), 22 ng. Chromatographic conditions as in Fig. 1.

RESULTS AND DISCUSSION

Owing to the complex composition of *O. spinosa* extracts, gradient elution was required to achieve adequate resolution of the flavonoids. The best resolution was obtained on C_{18} bonded column using the gradient described under *Elution* (Fig. 1).

A typical chromatogram of a synthetic mixture of rutin, genistein, kaempferol, formononetin, benzyl-4-hydroxybenzoate (internal standard) and biochanin A is shown in Fig. 2. Over a period of four months, retention times of compounds 1–6 varied only slightly and within the ranges 2.38–2.44, 16.82–16.91, 17.80–17.85, 19.09–19.15, 21.98–22.10 and 22.71–22.74 min, respectively. The resolved peaks were identified using the co-injection technique with authentic samples.

A series of standard samples (up to 200 ng) were prepared according to the procedure described under *Calibration graphs.*

Chromatographic results showed that the graphs of peak area ratio (genistein/internal standard, formononetin/internal standard and biochanin A/internal standard) *versus* the amount of genistein, formononetin and biochanin A injected are linear up to 200 ng. These linear relationships can be expressed by the following equations:

$y = 0.0266x \quad r = 0.998$ (genistein)
$y = 0.017x \quad r = 0.989$ (formononetin)
$y = 0.033x \quad r = 0.999$ (biochanin A)

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

TABLE I

CONTENTS OF GENISTEIN, FORMONONETIN AND BIOCHANIN A IN FOUR DIFFERENT SAMPLES OF *O. SPINOSA* ROOTS

Source of sample	*Genistein (mg/100 g)*	*Formononetin (mg/100 g)*	*Biochanin A (mg/100 g)*
Indena	0.8	4.3	0.15
Brisighello	6,6	5,1	0.20
Ulrich	5.3	6.3	0.22
Galke	0.3	3.1	0.06

Under the experimental conditions used, it was found that the lowest detectable amount for each flavonoid was 5 ng.

This HPLC method was used in the analysis of four samples of *O. spinosa* and the values obtained are given in Table I.

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