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Note

High-performance liquid chromatographic determination of flavonoid glucosides from *Helichrysum italicum*

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Helichrysum italicum G. Don (*H. angustifolium* D.C.) is a plant widely distributed in the Mediterranean area, and its flowering tops are used in folk medicine for their anti-inflammatory and anti-allergic properties [1]. The constituents of the drugs are terpenes, sterols [2] and flavonoids [3,4]. Thin-layer chromatography was recently utilized to isolate and identify flavonoid derivatives from the species *H. graveolens* [5]. However, high-performance liquid chromatography (HPLC) is the method of choice for the separation and quantification of complex natural mixtures of flavonoids [6]. Recently we reported that eluents containing C_3 alcohols yield better separations than those obtained with the customary acetonitrile (methanol)–water–acetic acid system [7]. According to this approach, an isocratic reversed-phase HPLC method for the separation of the main flavonoid glucosides of *H. italicum* has been developed, and the results are described in this paper.

EXPERIMENTAL

Materials

Naringenin-4′-glucoside, kaempferol-3-glucoside and 4,2′,4′,6′-tetrahydroxy-chalcone-2′-glucoside were isolated from *H. italicum* according to the literature [4]. The flowering tops of *H. italicum* were collected from the Ligurian mountains and authenticated at the Department of Botany (University of Milan). 2-Propanol was of HPLC grade (Baker, Deventer, The Netherlands).

Apparatus

The HPLC analyses were performed on a Waters Assoc. (Milford, MA, U.S.A.) 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,

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Waldbronn, F.R.G.). The column was C_8 Aquapore RP 300 (7 μm spherical) (250 × 4.6 mm I.D.) (Brownlee Labs., Santa Clara, CA, U.S.A.). The eluent was 2-propanol–water (20:80) at a flow-rate of 1.5 ml/min.

The acquisition of UV spectra was automatic at the apex, both inflection points and the base of all peaks (200–500 nm, 2-nm steps).

Purity of 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 to be chromatographically pure when there was exact coincidence among their corresponding UV spectra.

Sample preparation

The flowers of *H. italicum* (6 g) were extracted by percolation with 70% ethanol, the solvent was evaporated under vacuum and the residue dissolved in methanol (10 ml). After centrifugation, the methanol was evaporated to dryness under vacuum to give a brownish yellow crude extract (0.5 g). This was suspended in water (20 ml) and, after filtration, the aqueous solution was evaporated to yield the flavonoid glucosides fraction (*ca.* 130 mg).

For HPLC analysis, a methanolic solution (6.5 mg/ml) was prepared; 5-μl aliquots were injected.

Calibration graphs

Naringenin-4′-glucoside, kaempferol-3-glucoside and 4,2′,4′,6′-tetrahydroxychalcone-2′-glucoside were dissolved in methanol (0.5 mg/ml). Replicate injections of these solutions (2.5–15 μl) were made. UV detection was at 254 nm.

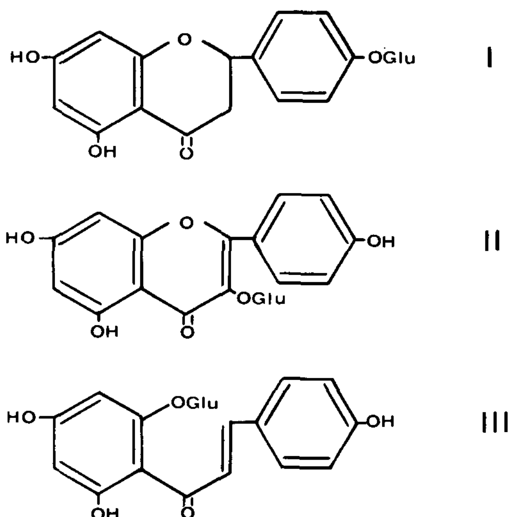

Fig. 1. Structures of main *Helichrysum italicum* flavonoid glucosides: naringenin-4′-glucoside (I), kaempferol-3-glucoside (II) and 4,2′,4′,6′-tetrahydroxychalcone-2′-glucoside (III).

RESULTS AND DISCUSSION

Naringenin-4′-glucoside (I), kaempferol-3-glucoside (II) and 4,2′,4′,6′-tetrahydroxychalcone-2′-glucoside (III) are the main flavonoids of *H. italicum* (Fig. 1). These compounds were isolated from *H. italicum* and their structures were confirmed by mass and NMR spectrometry [4]. For the separation of these flavonoids, a simple 20% isopropanol solvent as an alternative to the acetonitrile (methanol)–acetic acid–water system yielded very satisfactory results (Fig. 2).

A typical chromatogram obtained from the flavonoid fraction of *H. italicum* is shown in Fig. 3. The UV spectra acquired for each peak were computer normalized and the plots were superimposed, obtaining exact coincidence among the curves. Consequently, the peaks were assumed to be pure. Moreover, the UV spectra of the peaks of interest and those of their corresponding standards were identical, which confirmed the previous identification through the retention times.

Rectilinear responses between peak areas and amounts injected were obtained when replicate injections ($n = 6$) of I–III reference solutions (1–7.5 μg) were made. The relationships were as follows:

$y = 178x + 47$ $r = 0.999$ (naringenin-4′-glucoside)
$y = 900x + 56$ $r = 0.995$ (kaempferol-3-glucoside)
$y = 627x - 59$ $r = 0.998$ (4,2′,4′,6′-tetrahydroxychalcone-2′-glucoside)

where y = peak area and x = amount injected (μg).

Quantification in the flavonoid fraction was achieved by external standardization and the contents of naringenin-4′-glucoside, kaempferol-3-glucoside and 4,2′,4′,6′-tetrahydroxychalcone-2′-glucoside were 9.2, 3.1 and 1.6%, respectively.

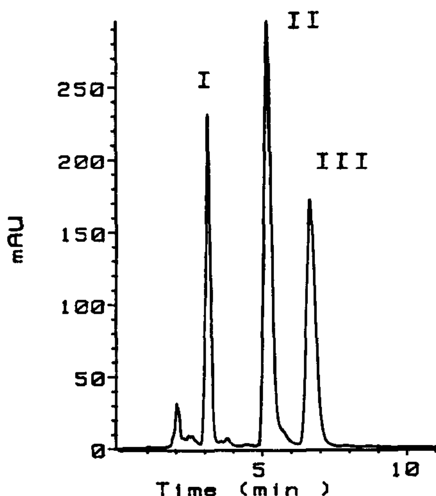

Fig. 2. High-performance liquid chromatogram of naringenin-4′-glucoside (I, 5 μg), kaempferol-3-glucoside (II, 3 μg) and 4,2′,4′,6′-tetrahydroxychalcone-2′-glucoside (III, 2 μg) standards. Column, 7-μm C_8 Aquapore RP 300; eluent, 2-propanol–water (20:80); flow-rate, 1.5 ml/min; detection, 254 nm.

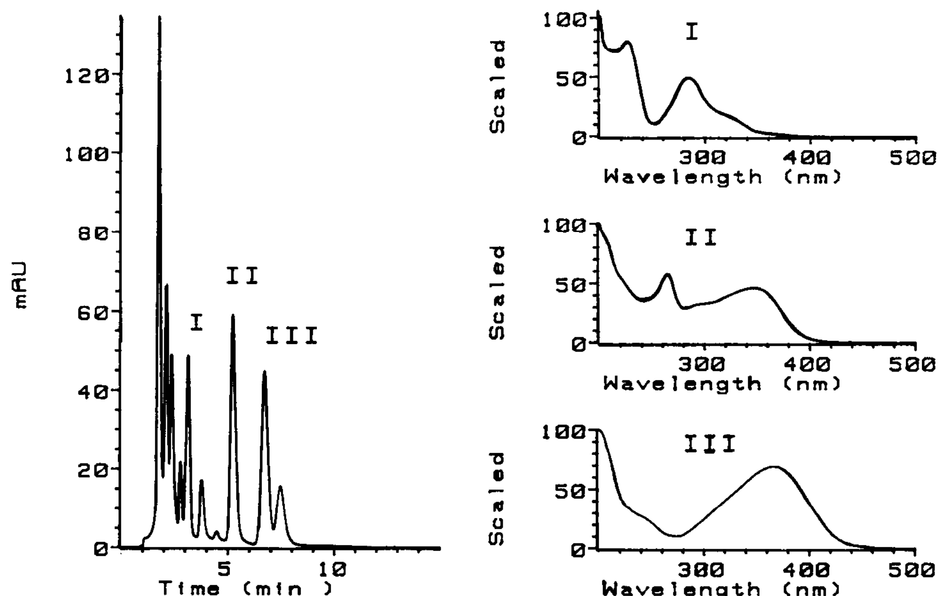

Fig. 3. Typical chromatogram of an *H. italicum* extract. See Fig. 2 for chromatographic conditions.

The reproducibility of the method was 98.0 ± 1.9% and recovery tests on known amounts of standards added to the extract gave 97.2 ± 2.5%. Intra-day and inter-day assay variations were 3.1% and 2.9%, respectively.

In conclusion, the described procedure provides further support for the validity of using eluents based on C_3 alcohols for the isocratic reversed-phase determination of flavonoids in medicinal plants.

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