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Short Communication

High-performance liquid chromatography with diode-array ultraviolet detection of methoxylated flavones in *Orthosiphon* leaves

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ABSTRACT

A rapid method for the determination of polymethoxylated flavones present in *Orthosiphon* leaves is described. Sinensetin, tetramethylscutellarein and 3'-hydroxy-4',5,6,7-tetramethoxyflavone were separated on a C_{18} 3-μm Microsorb column using 2-propanol–tetrahydrofuran–water (22:4:74) as the eluent, followed by diode-array UV detection. The procedure was also applicable to the determination of polymethoxylated flavones from orange peel.

INTRODUCTION

Flavonoids have been shown to be ubiquitous in plants [1] and their chemistry and pharmacology are well documented [2,3]. Among them the polymethoxylated flavones are notable because they constitute a special group found in *Orthosiphon spicatus* (Thunb.) leaves [4,5] and in some citrus species [6].

Polymethoxylated flavones have often been examined in the past by spectrophotometric [7] or liquid chromatographic methods [8]. Several high-performance liquid chromatographic (HPLC) methods, mainly based on gradient elution, have been described for their determination in citrus and orange juices. So far no report has appeared on the HPLC of the polymethoxylated flavones present in *Orthosiphon* leaves. In this study, a rapid method for the determination of these flavones is described. Separation was achieved by reversed-phase isocratic elution with 2-propanol–tetrahydrofuran–water (22:4:74), followed by diode-array UV detection (DAD).

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EXPERIMENTAL

Materials

Orthosiphon spicatus leaves were obtained from Milanfarma (Milan, Italy). 3′,4′,5,6,7-pentamethoxyflavone (sinensetin, **I**), 3′,5-dihydroxy-4′,6,7-trimethoxyflavone (eupatorin, **IV**) and 4′,5,6,7,8-pentamethoxyflavone (tangeretin, **VII**) were purchased from Extrasynthese (Genay, France). 4′,5,6,7-Tetramethoxyflavone (tetramethylscutellarein, **III**), 3′,4′,5,6,7,8-hexamethoxyflavone (nobiletin, **V**) and 3′,4′,3,5,6,7,8-heptamethoxyflavone (**VI**) were already available in our laboratory [9].

Chromatographic conditions

The HPLC system consisted of a Model 510 pump, equipped with a Model U6K universal injector and a Model 1040 photodiode-array detector (Hewlett-Packard, Waldbronn, Germany). Chromatographic runs were performed on a 3-μm C_{18} Microsorb column (100 mm × 4.6 mm I.D.) (Rainin, Woburn, MA, USA). The mobile phase was 2-propanol–tetrahydrofuran–water (22:4:74) at a flow-rate of 0.5 ml/min (*Orthosiphon*) or 0.6 ml/min (orange peel).

Sample preparation

A 1-g amount of powdered *Orthosiphon* leaves was extracted by shaking for 10 min with 10 ml of methylene chloride. The clear filtrate (Schleicher and Schüll, folded filters) was evaporated to dryness *in vacuo* and the residue was dissolved in 1 ml of methanol. The solution was filtered through Spartan 13 filters (0.45 μm) and 10–20 μl were injected.

A 1-g amount of orange peel was extracted by shaking for 15 min with 10 ml of methanol. The clear filtrate was evaporated to dryness and the residue was dissolved in 2 ml of 30% methanol and percolated under positive pressure through a Sep-Pak C_{18} cartridge (Waters Assoc.) previously activated with methanol (3 ml) and water (5 ml). After washing with 3 ml of 50% methanol, the polymethoxylated flavones fraction was eluted with 3 ml of 70% methanol. Volumes of 100–200 μl were injected.

Evaluation of peak purity

To check peak purity, the eluates were monitored with the photodiode-array detector (200–400 nm). The three spectra corresponding to the upslope, apex and downslope of each peak were normalized and superimposed. Peaks were considered pure when there was exact coincidence between the three spectra (match factor >995).

RESULTS AND DISCUSSION

A number of lipophilic flavonoids are present in *Orthosiphon* leaves, sinensetin and tetramethylscutellarein being the most abundant. In addition to these two components, orange peel contains nobiletin, 3′,4′,3,5,6,7,8-heptamethoxyflavone and tangeretin [10] (Fig. 1). It should be noted that polymethoxylated flavones differ only in the position and the number of methoxy groups. Owing to the hydrophobic nature of these compounds and the small difference in polarity, C_{18} columns were used with eluents containing 2-propanol and tetrahydrofuran, whose suitability in resolving

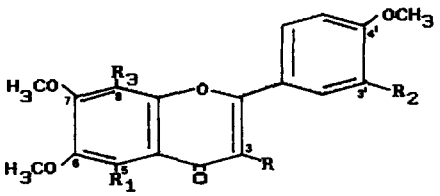

Fig. 1. Polymethoxylated flavones of *Orthosiphon spicatus* leaves and orange peel. R_1, R_2 = OCH_3; R, R_3 = H: sinensetin (**I**). R_1 = OCH_3; R, R_2, R_3 = H: tetramethylscutellarein (**II**). R_1 = OCH_3; R_2 = OH; R, R_3 = H: 3'-hydroxy-4',5,6,7-tetramethoxyflavone (**III**). R_1, R_2 = OH; R, R_3 = H: eupatorin (**IV**). R_1, R_2 = OH; R, R_3 = H: nobiletin (**V**). R, R_1, R_2, R_3 = OCH_3: 3',4',3,5,6,7,8-heptamethoxyflavone (**VI**). R_1, R_3 = OCH_3; R, R_2 = H: tangeretin (**VII**).

flavonoids has already been ascertained [11]. The isocratic mobile phase composition was 2-propanol–tetrahydrofuran–water (22:4:74), giving a sharp baseline resolution in less than 25 min (Fig. 2).

Different apolar solvents were evaluated for their ability to extract selectively polymethoxylated flavonoids from *Orthosiphon* leaves. Methylene chloride was selected, as the flavonoids were extracted with a very small amount of relatively polar compounds, which did not interfere with the early portion of the chromatogram. For this reason, further purification using Sep-Pak C_{18} was unnecessary. The UV spectra scanned from the peaks of eluting standards were in agreement with those from the peaks eluting at the corresponding times in the leaves sample.

Linearity between polymethoxylated flavone concentration and peaks area was found between 10 and 50 μg/ml, the correlation coefficients being 0.997 and 0.995 for sinensetin and tetramethylscutellarein, respectively. Recovery tests with known

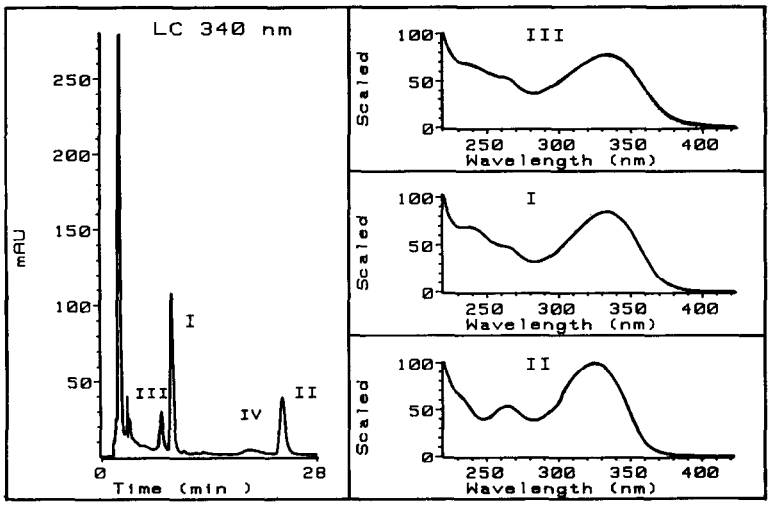

Fig. 2. Chromatogram obtained from *Orthosiphon* leaves. Chromatographic conditions: column, 3-μm C_{18} Microsorb; eluent, 2-propanol–tetrahydrofuran–water (22:4:74); flow-rate, 0.5 ml/min; UV detection at 340 nm. For peaks, see Fig. 1.

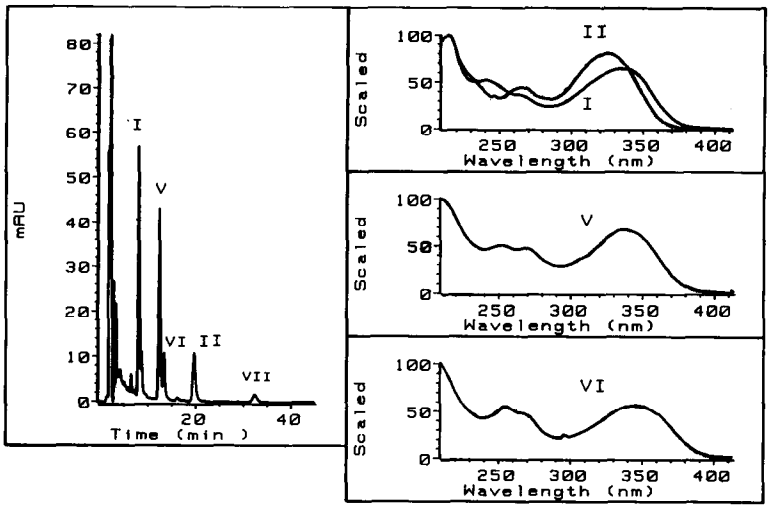

Fig. 3. Chromatogram obtained from orange peel. Chromatographic conditions as in Fig. 2. Flow-rate, 0.6 ml/min. For peaks, see Fig. 1.

amounts of standards added to a sample were performed, and the recoveries were in the range 92–108% (n = 6; S.D. = 4.9%).

This HPLC procedure was also applied to the determination of polymethoxylated flavones in orange peel. Prepurification of the sample through a Sep-Pak C_{18} cartridge was essential in this instance to obtain a satisfactory separation of sinensetin, heptamethoxyflavone, tetramethylscutellarein and tangeretin (Fig. 3).

The presence of chlorophylls and carotenoids in *Orthosiphon* leaves and orange peel was excluded on the basis of both sample purification and diode-array analysis.

In conclusion, the described method for the determination of polymethoxylated flavones in *Orthosiphon* leaves offers several advantages over existing thin-layer chromatographic methods. Moreover, it can be applied satisfactorily to the determination of analogues flavonoids in other matrices, such as orange peel.

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