Journal of Chromatography, 593 (1992) 165–170
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2411

Separation of flavonol-2-O-glycosides from *Calendula officinalis* and *Sambucus nigra* by high-performance liquid and micellar electrokinetic capillary chromatography

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ABSTRACT

Calendula officinalis and *Sambucus nigra* flowers were analysed by reversed-phase high-performance liquid chromatography (RP-HPLC) and micellar electrokinetic capillary chromatography (MECC). RP-HPLC was performed on C_8 Aquapore RP 300 columns with eluents containing 2-propanol and tetrahydrofuran. MECC was carried out on a 72-cm fused-silica capillary using sodium dodecyl sulphate and sodium borate (pH 8.3) as the running buffer. The results obtained by these techniques are compared.

INTRODUCTION

Flavonoids are a widespread group of natural products and in recent years different investigations on their biochemical and pharmacological properties have been reported [1].

Flavonoids are mainly described for their ability to inhibit a variety of enzymes [2] and for their radical scavenging [3] and anti-inflammatory [4] activity. Their analysis is therefore of prime importance, and so far high-performance liquid chromatography (HPLC) has been the main method used.

According to our strategy [5], flavonoids from *Betula folium, Ononis spinosa, Helichrysum italicum, Ginkgo biloba, Anthemis nobilis, Equisetum arvense, Orthosiphon spicatus and Solidago virgaurea* can be sharply separated by elution on C_8 columns with systems containing 2-propanol and tetrahydrofuran. Recently [6], we reported the separation of flavonol-3-O-glycosides from *Ginkgo biloba* by micellar electrokinetic capillary chromatography (MECC). Extending this investigation, in this work *Calendula officinalis* and *Sambucus nigra* flowers were analysed by RP-HPLC and MECC.

EXPERIMENTAL

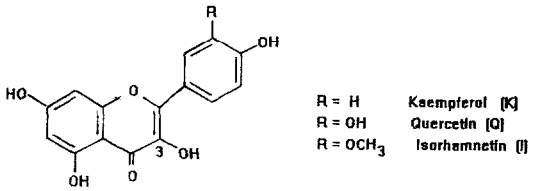
Materials

The reference compounds a, b, e and h (Table I) were purchased from Extrasynthese (Genay, France). Astragalin (i) was already available in this laboratory [7]. *Calendula officinalis* and *Sambucus nigra* flowers were obtained from Milanfarma (Milan, Italy). All other chemicals were of HPLC grade.

0021-9673/92/$05.00

TABLE I

FLAVONOL-3-O-GLYCOSIDES FROM *CALENDULA OFFICINALIS* AND *SAMBUCUS NIGRA*



Compound	Peak
I-3-O-glucoside	a
I-3-O-6-rhamnosylglucoside (I-3-O-rutinoside)	b
I-3-O-2-rhamnosylglucoside (I-3-O-neohesperidoside)	c
I-3-O-2^G-rhamnosylrutinoside	d
Q-3-O-6-rhamnosylglucoside (rutin)	e
Q-3-O-2-rhamnosylglucoside (Q-3-O-neohesperidoside)	f
Q-3-O-2^G-rhamnosylrutinoside	g
Q-3-O-glucoside (isoquercitrin)	h
K-3-O-glucoside (astragalin)	i

Equipment

HPLC. HPLC analyses were performed on a Waters Assoc. (Milford, MA, USA) 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, Germany). The analytical column was a 7-μm C_8 Aquapore RP 300 cartridge (220 × 2.1 mm) and the semi-preparative column was a 7-μm C_8 Aquapore RP 300 (250 × 7 mm I.D.) (Applied Biosystems, San Jose, CA, USA).

The eluent for *Calendula officinalis* flowers was 2-propanol–tetrahydrofuran–water (10:5:85) at a flow-rate of 0.4 ml/min [in semi-preparative runs, the flow-rate was 4 ml/min and the peaks were collected by means of a Gilson Model 201 fraction collector (Biolabo Instruments, Milan, Italy)]. The eluent for *Sambucus nigra* flowers was 2-propanol–tetrahydrofuran–water (12:4:84) at a flow-rate of 0.4 ml/min.

MECC. The separations were performed using a Applied Biosystems Model 270A capillary electrophoresis apparatus equipped with a 72 cm × 50 μm I.D. fused-silica capillary. The buffer was 20–25 m*M* sodium borate (pH 8.3) and 40–60 m*M* sodium dodecyl sulphate (SDS). The other conditions were voltage 277 V/cm, temperature 27°C, injection 1-s aspiration (4 nl) and detection at 260 and 320 nm. The data were analysed on a Shimadzu (Kyoto, Japan) CR3A data processor.

Sample preparation

Calendula officinalis. Dried flowers (2 g) were extracted with 20 ml of 50% methanol at room temperature for 24 h. The clear filtrate was evaporated to dryness under vacuum and the residue was dissolved in 3 ml of methanol. A 1-ml volume of this solution was diluted with 2 ml of water and applied to a previously activated (5 ml of methanol followed by 5 ml of water) Sep-Pak C_{18} cartridge. After washing with 3 ml of water and 3 ml of 30% methanol, the flavonoid fraction was eluted with 5 ml of 70% methanol. The eluate was evaporated to dryness and the residue was dissolved in 1 ml of 30% methanol.

Sambucus nigra. Dried flowers (2.5 g) were extracted with 50 ml of 50% methanol at room temperature for 48 h. After filtration, the solution was evaporated to dryness under vacuum and the residue was dissolved in 2 ml of methanol. A 0.5-ml volume of this solution was processed as described above.

Hydrolysis

Acid hydrolysis was carried out in sealed tubes at 100°C for 45 min with 0.5 mg of d glycoside dis-

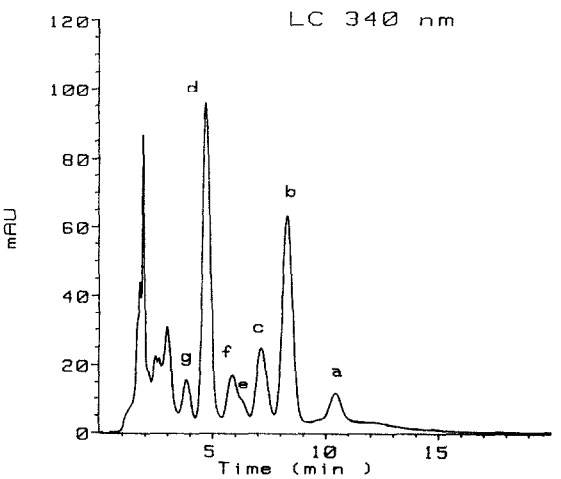


Fig. 1. Typical chromatogram of *Calendula officinalis* flower extract. Column, 7-μm C_8 Aquapore RP 300 cartridge (220 × 2.1 mm I.D.); eluent, 2-propanol–tetrahydrofuran–water (10:5:85); flow-rate, 0.4 ml/min. For peaks, see Table I.

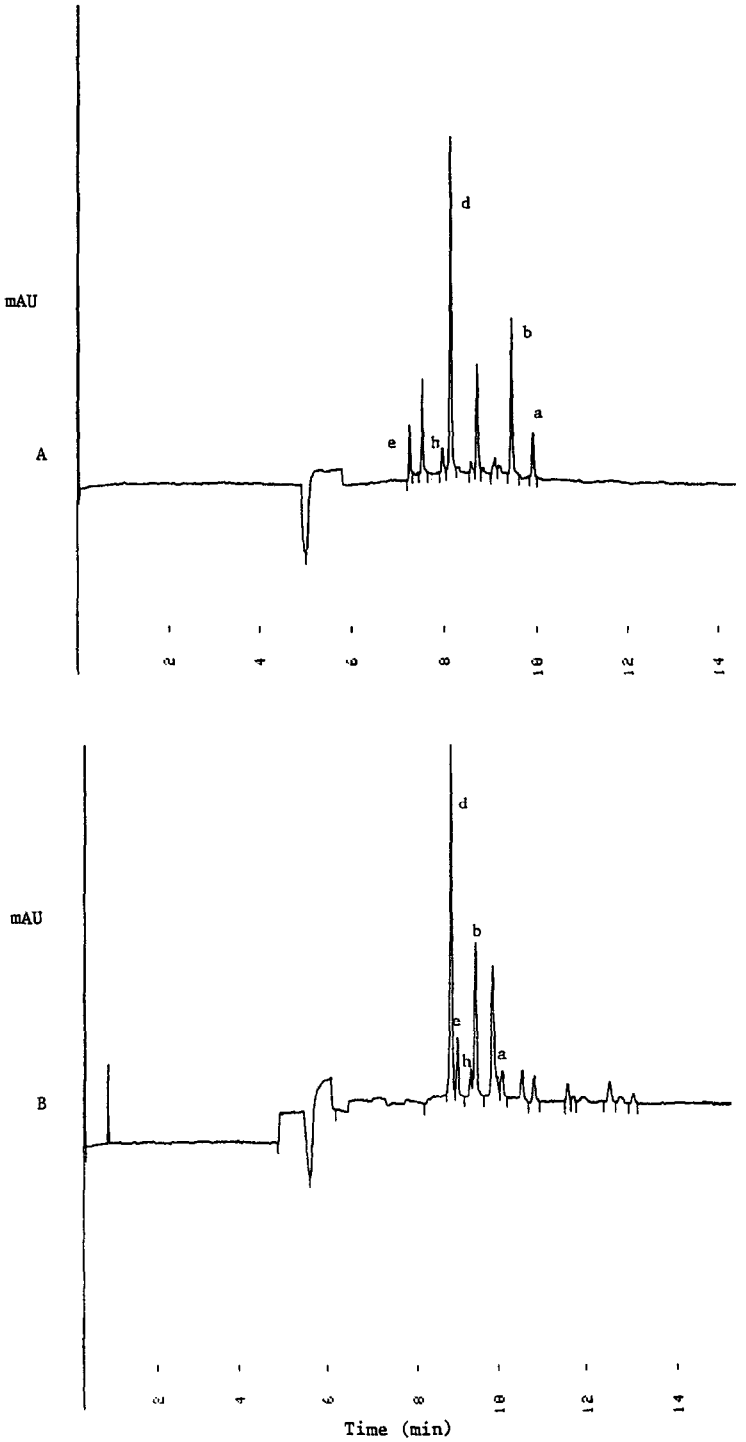


Fig. 2. Typical electropherograms of *Calendula officinalis* flower extract. Conditions: capillary 72 cm × 50 μm I.D. fused silica; voltage, 277 V/cm; buffer, (A) 60 m*M* SDS–20 m*M* borate (pH 8.3) and (B) 40 m*M* SDS–25 m*M* borate (pH 8.3); detection, 260 nm; attenuation, 16 mV full-scale. For peaks, see Table I.

solved in 0.2 ml of *M* HCl and 0.8 ml of methanol. Isorhamnetin was detected by HPLC [8], whereas glucose and rhamnose were detected as acetyl derivatives by gas chromatography [9].

RESULTS AND DISCUSSION

Calendula officinalis

Different flavonol-3-O-glycosides have recently been identified in *Calendula officinalis* flowers [10], and their structures elucidated by NMR and mass spectrometry (Table I). A typical chromatogram of an extract is shown in Fig. 1. Peaks a and b were identified as isorhamnetin-3-O-glucoside and isorhamnetin-3-O-rutinoside, respectively, by co-chromatography with authentic specimens; moreover, the identity was confirmed by comparing their on-line UV spectra with those of corresponding standards. Also the peaks c and d gave spectra typical of isorhamnetin glycosides. The major peak d was isolated and on acid hydrolysis isorhamnetin and rhamnose and glucose (in the ratio 2:1) were detected, thus indicating that this peak was the previously reported isorhamnetin-3-O-2^G-rhamnosylrutinoside [10].

Owing to its chromatographic and spectrophotometric characteristics, peak c can be considered as the fourth described isorhamnetin derivative, *i.e.*, isorhamnetin-3-O-neohesperidoside. Peaks e, f and g gave one-line UV spectra typical of quercetin. Of these peaks, the low and partially resolved peak e was identified as rutin by co-chromatography with a standard. Peaks f and g can be assigned as the previously reported quercetin-3-O-neohesperidoside and quercetin-3-O-2^G-rhamnosylrutinoside on the basis of their chromatographic behaviour and UV spectra.

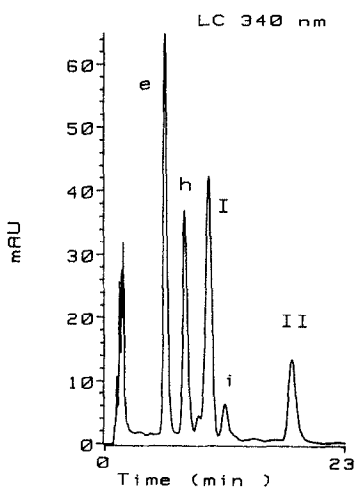


Fig. 3. Typical chromatogram of a *Sambucus nigra* flower extract. Column as in Fig. 1; eluent, 2-propanol–tetrahydrofuran–water (12:4:84); flow-rate, 0.4 ml/min. For peaks, see Table I.

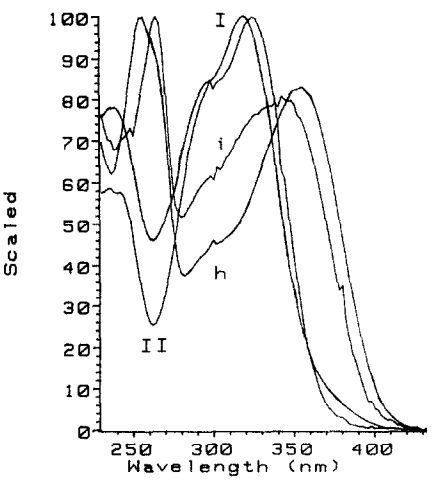


Fig. 4. UV spectra of isoquercitrin (h), astragalin (i) and peaks I and II in Fig. 3.

Typical electropherograms obtained from *Calendula officinalis* flower extract using two different running buffers are shown in Fig. 2. MECC allowed confirmation of the presence of rutin (peak e) and the detection of isoquercitrin (peak h). Analogously, peak a, b and d were identified by comparison with authentic specimens. Owing to the lack of on-line spectral information, the identification of the other three peaks was not possible.

Sambucus nigra

Rutin and isoquercitrin (Table I) are the main flavonolglycosides of *Sambucus nigra* flowers [11]. These compounds were sharply separated by isocratic eluton (Fig. 3) and their identities were confirmed by co-chromatography and on-line UV spectral comparison with rutin and isoquercitrin standards. A small peak due to astragalin was also identified. As shown in Fig. 4, peaks I and II gave UV

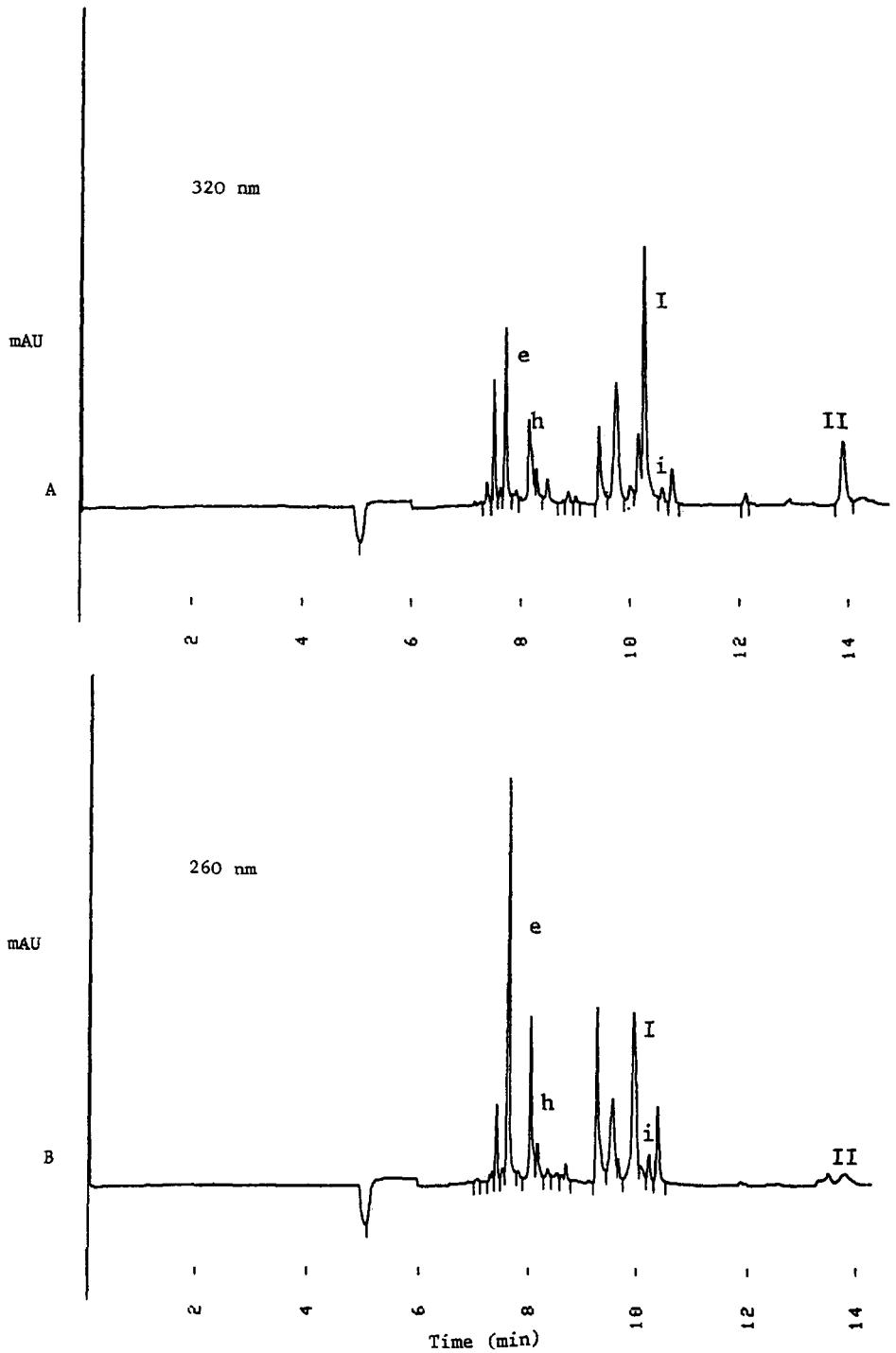


Fig. 5. Typical electropherograms of *Sambucus nigra* flower extract, with detection at (A) 320 and (B) 260 nm. Conditions as in Fig. 2A. For peaks, see Table I.

spectra with a minimum at 260 nm and a maximum near 320 nm, thus excluding a flavonol structure.

MECC of *Sambucus nigra* flowers extracts yielded a baseline separation within 12 min (Fig. 5). Peaks e, h and i were assigned by comparison with standards, whereas peaks I and II were identified by differential UV absorption at 260 and 320 nm.

From these results, it can be concluded that MECC can be used as a complementary technique with HPLC for the analysis of flavonol-3-O-glycosides from *Calendula officinalis* and *Sambucus nigra* flowers. However, technological improvement of capillary electrophoresis apparatus for on-line UV spectra is needed to achieve the same analytical data as HPLC.

ACKNOWLEDGEMENT

The authors are grateful to CNR–P.S. "Innovazione Produttiva nella P&MI" for providing funds.

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