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High-performance liquid chromatography and micellar electrokinetic chromatography of flavonol glycosides from *Tilia*

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ABSTRACT

The determination of nine different flavonol glycosides from *Tilia* using reversed-phase high-performance liquid chromatography (HPLC) and micellar electrokinetic chromatography (MEKC) is described. The analytes were monitored by on-line diode-array UV detection to identify peaks as quercetin or kaempferol derivatives. MEKC is confirmed as a useful complementary technique to HPLC.

INTRODUCTION

Tilia cordata and *Tilia phatyphyllos* have been used as sedatives in folk medicine. They are listed in several pharmacopoeias, and the presence of compounds that behave as ligands for brain benzodiazepine receptors has been described [1]. Most published analyses of *Tilia* flavonol glycosides have involved separations by TLC [2], where the resolution is not sufficient to detect unequivocally components with nearly identical R_F values. Wagner *et al.* [3] proposed a gradient HPLC method, but only quercitrin and tiliroside could be identified.

In this investigation, we applied a previously described HPLC approach [4], based on isocratic elution with 2-propanol and tetrahydrofuran (THF) using C_8 columns. The eluate was monitored by diode-array UV detection (DAD) to identify peaks as quercetin or kaempferol derivatives. Flavonol glycosides from *Tilia* were also analysed by micellar electrokinetic chromatography (MEKC) combined with DAD, which has recently been proposed [5] as a complementary technique to HPLC. The structures of the flavonoids analysed are shown in Fig. 1.

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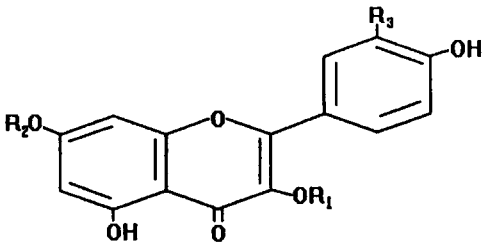


Quercetin-3-O-glucoside-7-O-rhamnoside (I)
R =glucose ; R =rhamnose ; R =OH
1 2 3
Kaempferol-3-O-glucoside-7-O-rhamnoside (II)
R =glucose ; R =rhamnose ; R =H
1 2 3
Quercetin-3,7-O-dirhamnoside (III)
R =R =rhamnose ; R =OH
1 2 3
Kaempferol-3,7-O-dirhamnoside (IV)
R =R =rhamnose ; R =H
1 2 3
Quercetin-3-O-glucoside (isoquercitrin) (V)
R =glucose ; R =H ; R =OH
1 2 3
Kaempferol-3-O-glucoside (astragalin) (VI)
R =glucose ; R =R =H
1 2 3
Quercetin-3-O-rhamnoside (quercitrin) (VII)
R =rhamnose ; R =H ; R =OH
1 2 3
Kaempferol-3-O-rhamnoside (VIII)
R =rhamnose ; R =R =H
1 2 3
Kaempferol-3-O-(6-p-coumaroyl)-glucoside (tiliroside) (IX)
R =6-p-coumaroyl-glucose ; R =R =H
1 2 3

Fig. 1. Structures of *Tilia* flavonol glycosides.

EXPERIMENTAL

High-performance liquid chromatography

HPLC analyses were performed using a Model 510 pump equipped with a Model U6K universal injector (Waters, Milford, MA, USA) and a model 1040 photodiode-array detector (Hewlett-Packard, Waldbronn, Germany). The column was MOS Hypersil (200 × 4.6 mm I.D.) and the eluent was 2-propanol–THF–water (10:5:85) at a flow-rate of 1.8 ml/min.

Micellar electrokinetic chromatography

MEKC separations were carried out using a Eureka 2000 CE–DAD apparatus (Kontron Instruments, Milan, Italy) equipped with a 72 cm × 75 μm I.D. fused-silica capillary. The running buffer was 30 m*M* sodium borate (pH 8.5)–50 m*M* sodium dodecyl sulphate (SDS). The voltage was 277 V/cm, the injection volume (by gravity) was 10 nl and the temperature was 27°C.

Materials

Tilia flowers were obtained from Milanfarma (Milan, Italy), Galke (Gittelde/Harz, Germany) and Birkenweg (Kleinesthein/Main, Germany).

Quercetin-3-O-glucoside (**V**), quercetin-3-O-rhamnoside (**VII**), myricetin-3-O-rhamnoside, kaempferol-3-O-glucoside (**VI**) and kaempferol-3-(*p*-coumaroyl)glucoside (**IX**) were purchased from Extrasynthese (Genay, France). Quercetin-3-O-glucoside-7-O-rhamnoside (**I**), kaempferol-3-O-glucoside-7-O-rhamnoside (**II**), quercetin-3,7-O-dirhamnoside (**III**) and kaempferol-3,7-O-dirhamnoside (**IV**) were obtained in our laboratory from *Tilia* leaves by semi-preparative HPLC.

2-Propanol, THF and water were of HPLC grade (Chromasolv, Riedel-de Haën, Hannover, Germany).

Sample preparation

Tilia powdered samples (flowers, leaves and herb) (2 g) were suspended in 50% methanol (20 ml) and left overnight at room temperature. After filtration, the solution was evaporated to dryness *in vacuo* and the residue was dissolved in methanol (2 ml).

Isolation of quercetin-3-O-glucoside-7-O-rhamnoside (**I**), kaempferol-3-O-glucoside-7-O-rhamnoside (**II**), quercetin-3,7-O-dirhamnoside (**III**) and kaempferol-3,7-O-dirhamnoside (**IV**). Aliquots of 50 μl of the sample solution were chromatographed on an Aquapore C_8 (7 μm) semi-preparative column (250 × 7 mm I.D.) using 2-propanol–THF–water (10:5:85) at a flow-rate of 4 ml/min. Peaks of **I–IV** were collected by means of a Gilson Model 201 fraction collector (Biolabo Instruments, Milan, Italy). Each run yielded 20–30 μg of each compound.

Hydrolysis. Aliquots of about 200 μg of **I–IV** were hydrolysed and the resulting aglycones were identified as described previously [6].

Tilia (flowers, leaves, herb) sample solutions (0.25 ml) were processed in an analogous manner to obtain the total amount of aglycones.

Glucose and rhamnose were detected by gas chromatography as acetyl derivatives [7].

Semi-preparative isolation of kaempferol-3-O-(6-p-coumaroyl)glucoside (tiliroside) (**IX**). A

1-ml volume of the sample solution was diluted to 3 ml with 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, tiliroside was eluted with 3 ml of methanol.

RESULTS AND DISCUSSION

Previous work [4] on the isolation of flavonoids from officinal plants involved extraction with aqueous methanol or ethanol followed by clean-up through a Sep-Pak C_{18} cartridge. Owing to the absence in *Tilia* of components eluting as a large front, solid-phase extraction was unnecessary, except for the semi-preparative isolation of tiliroside (**IX**).

Using 2-propanol–THF–water (10:5:85) in the isocratic mode, a baseline resolution of all the components was achieved in about 30 min. As shown in Fig. 2A, *Tilia* flowers contain isoquercitrin (**V**), astragalin (**VI**), quercitrin (**VII**), kaempferol-3-O-rhamnoside (**VIII**) and tiliroside (**IX**), the first being the major component. On the other hand, the main flavonols of *Tilia* leaves were the diglycosides **I–IV** and tiliroside, whereas **V–VIII** were present in smaller amounts (Fig. 2B). A chromatogram of *Tilia* herb is shown in Fig. 2C.

Peaks were identified as quercetin (Q), kaempferol (K) or kaempferol-*p*-coumaroyl (T) derivatives on the basis of their on-line UV spectra (Fig. 3). Thus, peaks I, III and II, IV were recognized from the diode-array spectra as quercetin and kaempferol derivatives, respectively. Each peak was isolated and subjected to acid hydrolysis followed by detection of the aglycone by HPLC and sugars by GC. As expected, peaks I and III yielded quercetin, whereas peaks II and IV produced kaempferol. The detected sugars were glucose and rhamnose in the ratio 1:1 for peaks I and II and rhamnose for peaks III and IV.

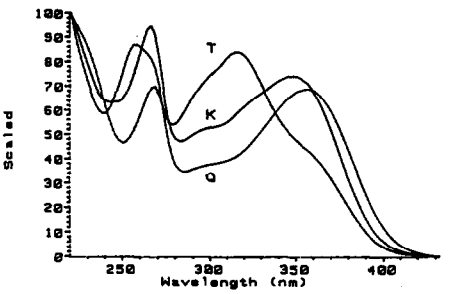


Fig. 3. HPLC–DAD of quercetin (Q), kaempferol (K) and kaempferol-*p*-coumaroyl (T) derivatives.

From these results it can be concluded that peaks I, III and IV are related to the previously reported [8] flavonol glycosides, and peak III may be reasonably assumed to be the analogue quercetin-3,7-dirhamnoside. Peaks V, VI, VII and IX were assigned by co-chromatography with standards, and their identities were confirmed by DAD.

The total amount of quercetin and kaempferol

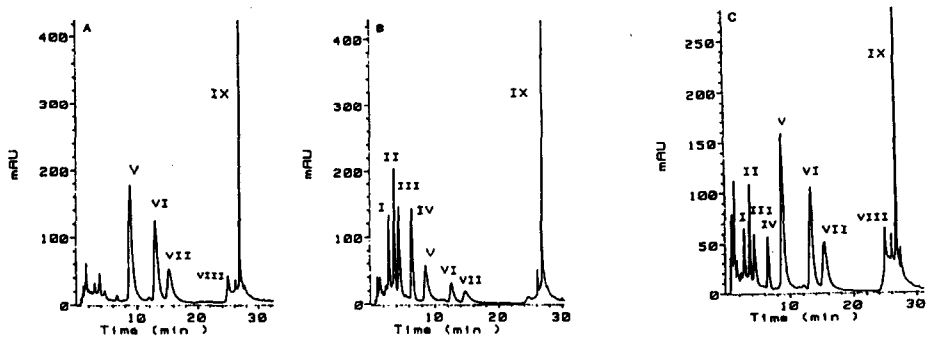
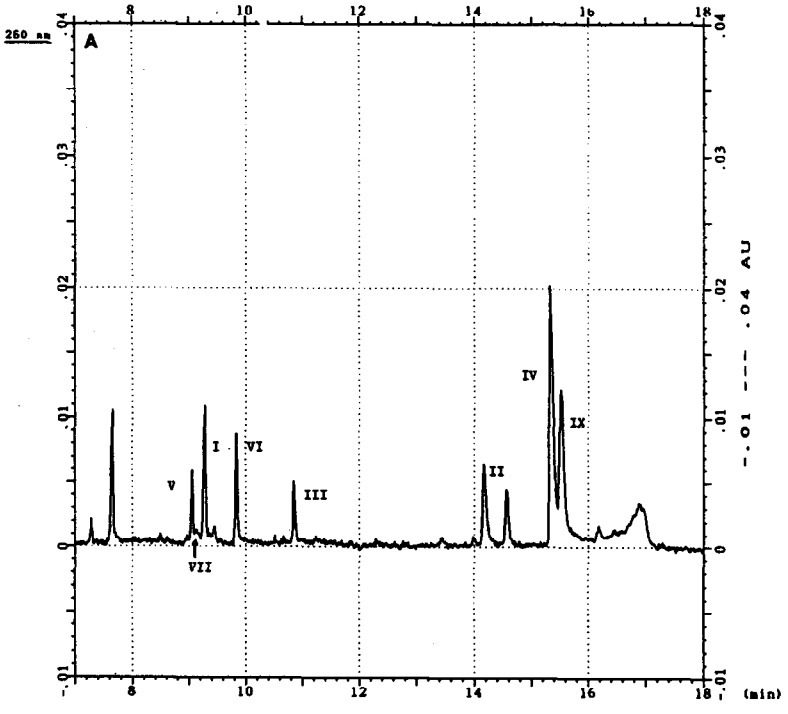


Fig. 2. Typical HPLC traces of (A) *Tilia* flowers, (B) *Tilia* leaves and (C) *Tilia* herb. Column, MOS Hypersil (200 × 4.6 mm I.D.); eluent, 2-propanol–tetrahydrofuran–water (10:5:85); flow-rate, 1.8 ml/min; detection, 270 nm. For peak numbers, see Fig. 1.



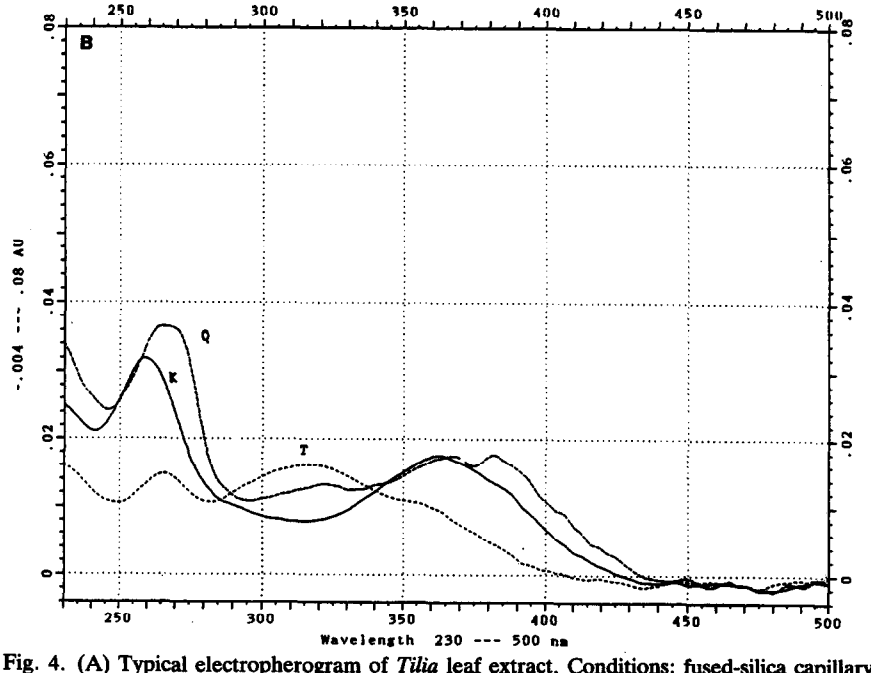


Fig. 4. (A) Typical electropherogram of *Tilia* leaf extract. Conditions: fused-silica capillary (72 cm × 75 μm I.D.); voltage, 277 V/cm; detection, 270 nm; buffer, 30 m*M* borate (pH 8.5)–50 m*M* SDS. (B) MEKC–DAD spectra of quercetin (Q), kaempferol (K) and kaempferol-*p*-coumaroyl (T) derivatives.

TABLE I

TOTAL AMOUNTS OF QUERCETIN AND KAEMPFEROL IN *Tilia* SAMPLES

Tilia	Sample	Quercetin in 250 mg of drug (μg)	Kaempferol in 250 mg of drug (μg)
Flowers	a	2.21	4.54
	b	2.59	3.21
	c	2.44	5.06
Leaves	a	0.37	1.39
	b	0.44	1.47
	c	0.58	1.82
Herb	a	2.90	5.59
	b	3.49	4.41
	c	3.18	5.12

derivatives in each sample was obtained by acid hydrolysis and HPLC determination of the resulting aglycones, and the results are given in Table I.

MEKC–DAD of *Tilia* leaf extracts yielded a baseline separation within 16 min (Fig. 4A). Peaks were identified by comparison with authentic specimens and on the basis of their on-line UV spectra (Fig. 4B). The wavelengths of the maximum absorption at around 265 nm of each compound are different from those shown in Fig. 3. This difference is ascribed to the different solvents [borate–SDS (pH 8.5) buffer in MEKC and 2-propanol–THF–water in HPLC] [9].

From these results it can be concluded that MEKC is a valuable alternative to HPLC and recent technological improvements in capillary electrophoresis apparatus for ultraviolet DAD permits on-line spectral information similar to that achieved by HPLC–DAD to be obtained.

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