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Identification of flavonoids from *Ginkgo biloba* L., *Anthemis nobilis* L. and *Equisetum arvense* L. by high-performance liquid chromatography with diode-array UV detection

PIERGIORGIO PIETTA*, PIERLUIGI MAURI and ANNAMARIA BRUNO
Università degli Studi di Milano, Via Celoria 2, 20133 Milan (Italy)
ANGELO RAVA
Istituto Biochimico Pavese, Viale Certosa 10, 27100 Pavia (Italy)
and
ENRICO MANERA and PIERLUIGI CEVA
SIT, via Cavour 57, 27032 Mede (Italy)

ABSTRACT

Naturally occurring flavonoids can be separated by reversed-phase high-performance liquid chromatography (HPLC) using 2-propanol and tetrahydrofuran. A development of this approach is described for the HPLC of *Ginkgo biloba*, *Anthemis nobilis* and *Equisetum arvense*. Peaks related to previously reported compounds were identified by co-chromatography with authentic standards and/or by diode-array detection. Tentative assignments of unknown peaks are presented.

INTRODUCTION

Naturally occuring flavonoids (aglycones and glycosides) can be separated and identified using high-performance liquid chromatography (HPLC), particularly reversed-phase HPLC. The separations are rapid and provide high resolution and sensitivity. Because of the analogous structure of most of the investigated compounds, relatively similar solvent mixtures are required to yield satisfactory separations. Methanol–water and acetonitrile–water are the preferred solvent systems.

The addition of acetic or formic acid to these eluents has been described in several papers [1], and frequently sharp separations are achieved only by using gradient elution [2]. Recently, [3] we reported that flavonoids of several medicinal plants can be sharply separated by isocratic elution on C_8 columns with systems containing C_3 alcohols and cyclic ethers. The validity of these systems has been demonstrated for different medicinal plant extracts such as *Betula* [4], *Ononis spinosa* [5] and *Helichrysum italicum* [6]. The present investigation demonstrates that these eluents can separate the flavonols of *Ginkgo biloba*, *Anthemis nobilis* and *Equisetum arvense*. In addition, the use of diode-array detection permits the peaks in the chromatogram to be assigned to previously reported compounds and tentative assignments of unknown peaks to be made.

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EXPERIMENTAL

Materials

2-Propanol and tetrahydrofuran (THF) were of analytical-reagent grade. *Ginkgo biloba* L. leaves, *Anthemis nobilis* L. flowers and *Equisetum arvense* L. leaves were obtained from different commercial sources. A standardized *Ginkgo biloba* extract was purchased from Laboratoires IPSEN. Authentic samples of isoquercitrin, luteolin-7-glucoside, quercitrin, apigenin-7-glucoside, apiin, rutin, kaempferol-3-rutinoside, isorhamnetin-3-rutinoside and sciadopitysin were obtained from Extrasynthese (Genay, France). Astragalin was isolated from *Helichrysum italicum* [6]; bilobetin, isoginkgetin and ginkgetin were prepared from *Ginkgo biloba* leaves [7]. Reference solutions were prepared in methanol (2 mg/ml).

Chromatographic conditions

The liquid chromatograph consisted of a Model U6K universal injector, two Model 510 pumps, a Model 680 automated gradient controller (all from Waters Assoc., Milford, MA, U.S.A.) and a Model HP 1040A photodiode-array detector (Hewlett-Packard, Waldbronn, Germany). The column was C_8 Aquapore RP 300 (250 mm × 4 mm I.D.) (Brownlee Labs., Santa Clara, CA, U.S.A.) with 7-μm spherical particles.

The eluents were as follows: for *Ginkgo biloba*, eluent A water–2-propanol (95:5), eluent B 2-propanol–THF–water (40:10:50), linear gradient from 20% to 60% B in 40 min, flow-rate 2 ml/min; and for *Anthemis nobilis* and *Equisetum arvense*, 2-propanol–water (15:85), flow-rate 2 ml/min.

The acquisition of UV spectra was automatic at the apex, both inflection points and base of all peaks (230–430 nm, 2-nm steps). Peaks of interest were collected by means of a Model 201 fraction collector (Gilson, Biolabo Instruments, Milan, Italy). Hydrolysis of the isolated glycosides were carried out according to the literature [8]. Glucose and rhamnose were detected by gas chromatography [9] as acetyl derivatives.

Sample preparation

Dried *Ginkgo biloba* leaves (600 mg) were extracted for 15 min under reflux with 60% aqueous acetone (15 ml) and the mixture was diluted to 25 ml with the same solvent and filtered through a fritted glass funnel. Prior to injection (5–10 μl), the samples were filtered through a 0.45-μm membrane filter.

Dried *Anthemis nobilis* flowers and *Equisetum arvense* leaves (1 g) were extracted with 15 ml of 60% methanol for 15 min at about 60°C, the clear filtrate was evaporated to dryness under vacuum and the residue was dissolved in 2 ml of methanol. A 1-ml volume of this solution was diluted with 2 ml of water and percolated through a Sep-Pak C_{18} cartridge (preactivated by passing 5 ml of methanol followed by 5 ml of water). After washing with 3 ml of water and 3 ml of 25% methanol, the flavonoid fraction was eluted with 6 ml of 70% methanol. The solvent was evaporated to dryness and the residue was dissolved in 1 ml of methanol. Aliquots of 10–20 μl were injected into the HPLC system.

Purity assay of the chromatographic peaks

The UV spectra of each peak, after subtraction of the corresponding UV base

spectrum, were computer normalized and the plots were superimposed. Peaks were considered to be homogeneous when there was exact correspondence among the corresponding spectra (match factor >990).

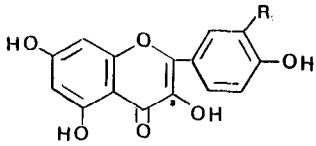
RESULTS AND DISCUSSION

Ginkgo biloba

The constituents of *Ginkgo biloba* leaves have been recently examined [10–12] and many flavonol glycosides have been identified (Table I).

TABLE I

FLAVONOL GLYCOSIDES OF *GINKGO BILOBA* LEAVES



R = OH Quercetin
R = H Kaempferol
R = OCH_3 Isorhamnetin

Type	Compound	No.
Quercetin-3-O-glycosides	Rutin (quercetin-3-O-rutinoside)	I
	Isoquercitrin (quercetin-3-O-glucoside)	II
	Quercitrin (quercetin-3-O-rhamnoside)	III
	Quercetin-3-O-[6″-*p*-coumaroylglucosyl-(1→2)rhamnoside]	IV
	Quercetin-3-O-rhamnosyl-(1→2)-rhamnosyl-(1→6)-glucoside	V
Kaempferol-3-O-glycosides	Kaempferol-3-O-rutinoside	VI
	Astragalin (kaempferol-3-O-glucoside)	VII
	Kaempferol-3-O-[6‴-*p*-coumaroylglucosyl-(1→2)-rhamnoside]	VIII
	Kaempferol-glycoside	IX
	Kaempferol-3-O-rhamnosyl-(1→2)-rhamnosyl-(1→6)-glucoside	X
Isorhamnetin-3-O-glycosides	Isorhamnetin-3-O-rutinoside	XI

The HPLC of a standardized extract of *Ginkgo biloba* leaves is shown in Fig. 1. Eleven major flavonoids were present (peaks I–XI). Six peaks were identified by comparison with authentic specimens (rutin, I; isoquercitrin, II; quercitrin, III; kaempferol-3-rutinoside, VI; astragalin, VII; and isorhamnetin-3-rutinoside, XI). Together with the retention time data, these peaks were also identified by comparing their UV spectra with those of the corresponding standards. The new spectra of peaks V and X presented a maximum slope in the short-wavelength region [band II, benzoyl typical of quercetin and kaempferol glycosides, respectively (Fig. 2)]. On acid hydrolysis of the isolated compounds, rhamnose and glucose in the ratio 2:1 were detected. These data combined with the short retention times (high polarity) indicated that peaks V and X were quercetin-3-O[rhamnosyl-(1→2)-rhamnosyl-(→6)-glucoside] and kaempferol-3-O-[rhamnosyl-(1→2)-rhamnosyl-(1→6)-glucoside], respectively. They have been previously reported in *Ginkgo biloba* [11,12].

Peak IX gave a spectrum similar to that of kaempferol-3-rutinoside (VI) (Fig. 3). Its resolution time (16 min) compared with that of kaempferol-3-rutinoside (12.3

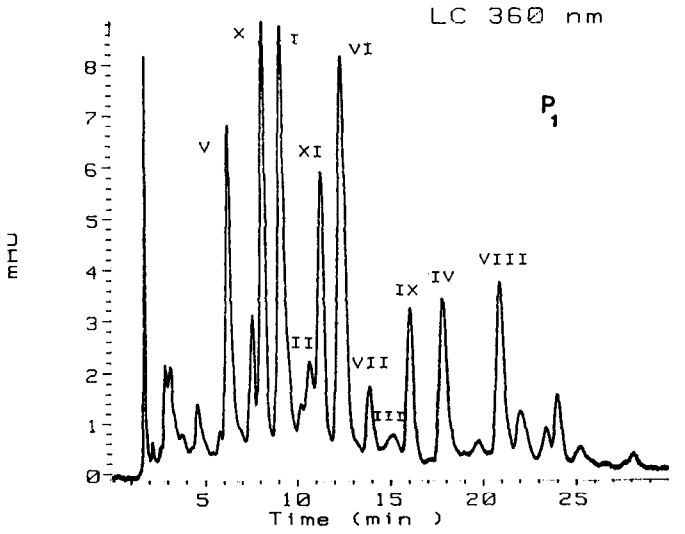


Fig. 1. Typical chromatogram of a standardized extract of *Ginkgo biloba* leaves. Column, 7-μm C_8 Aquapore RP-300; Eluent, (A) water–2-propanol (95:5), (B) 2-propanol–THF–water (40:10:50), linear gradient from 20% to 60% B in 40 min; flow-rate, 1 ml/min. For peaks, see Table I.

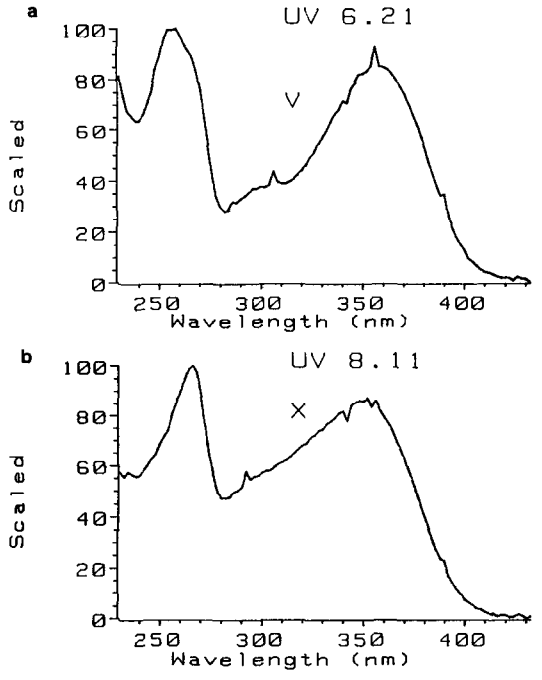


Fig. 2. UV spectra of (a) quercetin-3-O-[rhamnosyl-(1→2)-rhamnosyl-(1→6)-glucoside] (V) and (b) kaempferol-3-O-[rhamnosyl-(1→2)-rhamnosyl-(1→6)-glucoside] (X).

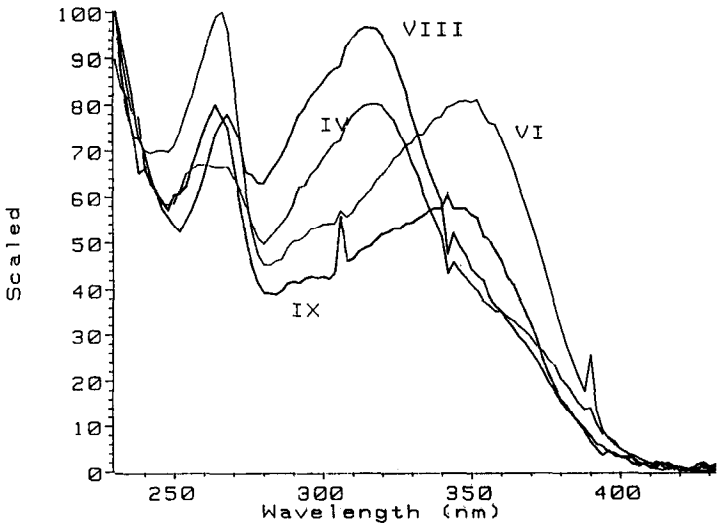


Fig. 3. UV spectra of kaempferol-3-O-rutinoside (VI), peak IX, quercetin-3-O-[6‴-*p*-coumaroylglucosyl-(1→4)-rhamnoside] (IV) and kaempferol-3-O-[6‴-*p*-coumaroylglucosyl-(1→4)-rhamnoside] (VIII).

min) demonstrated that it was less polar. As acid hydrolysis followed by gas chromatographic determination of the liberated sugars yielded glucose and rhamnose, peak IX might reasonably be assumed to be a glucosyl rhamnoside isomer. However, more work is needed for a complete structure elucidation. New peaks IV and VIII showed a band II absorption typical of quercetin and kaempferol glycosides, respectively (Fig. 3). The hypsochromic shift of band I (cinnamoyl ring, λ_{max} = 320 nm, instead of 350–360 nm) indicated the presence of the *p*-coumaroyl group in the side-chain. This finding was confirmed by HPLC determination of *p*-coumaric acid from the isolated peaks after alkaline hydrolysis. Hence, peaks IV and VIII were assigned to the previously identified [10] quercetin-3-O-[6‴-*p*-coumaroylglucosyl-(1→4)-rhamnoside] and kaempferol-3-O-[6‴-*p*-coumaroylglucosyl-(1→4)-rhamnoside], respectively.

A typical chromatogram obtained from *Ginkgo biloba* leaves is shown in Fig. 4. The chromatogram was divided into two parts on the basis of retention times: the first part (7–25 min, similar to that of Fig. 1 and indicated as P_1) consisted of the heterosides I–XI; the second part (P_2) contained the biflavones bilobetin, ginkgetin, isoginkgetin and sciadopitysin. These last were almost absent in the standardized extract.

Anthemis nobilis and Equisetum arvense

Apigenin-7-glucoside, luteolin-7-glucoside and apiin (apigenin-7-apioglucoside) are the characteristic components of *Anthemis nobilis* flowers [13] and isoquercitrin and quercetin-3-sophoroside are the main constituents of european *Equisetum arvense* [14] (Table II). Well resolved peaks were obtained from extracts of these plants by isocratic elution with 2-propanol–water (15:85). The identity of the peaks from *Anthemis nobilis* L. (Fig. 5) was established by co-chromatography using standard apiin, apigenin-7-glucoside and luteolin-7-glucoside.

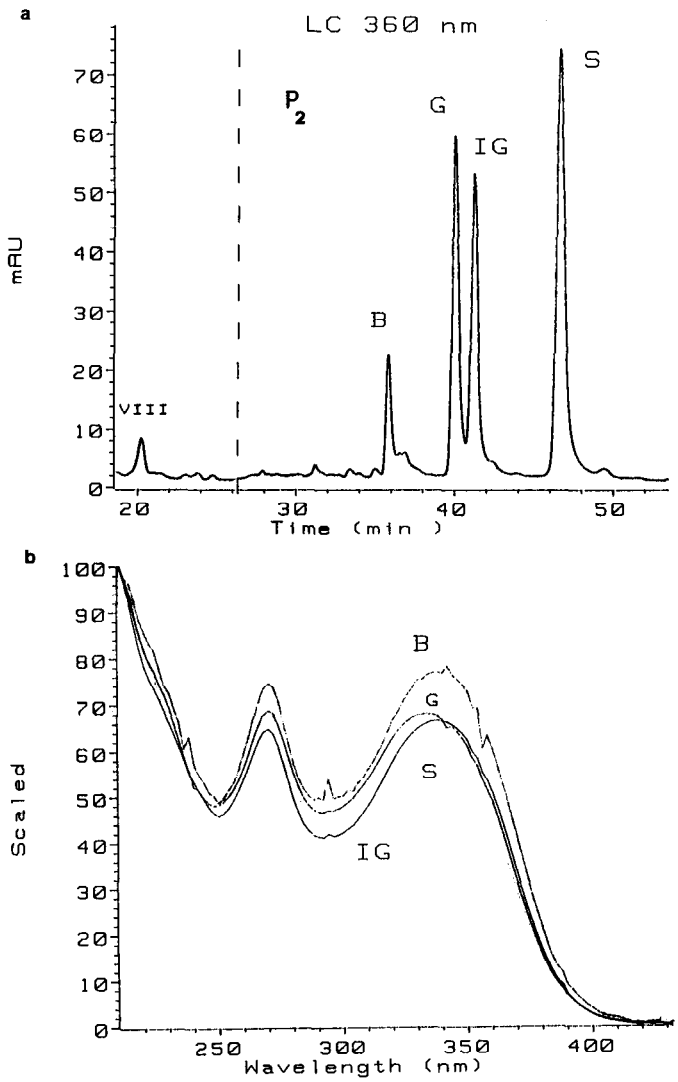
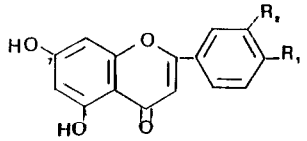


Fig. 4. Typical chromatogram from *Ginkgo biloba* leaves. (a) P_2 (b) UV spectra of bilobetin (B), ginkgetin (G), isoginkgetin (IG) and sciadopitysin (S). See Fig. 1 for P_1 and chromatographic conditions. Linear gradient from 20% to 80% in 60 min.

For *Equisetum arvense* L. (Fig. 6), only the main peak (8.65 min) and the small peak at 14.6 min were identified by co-chromatography with isoquercitrin and astragalin standards, respectively. The other peak (4.71 min) (whose UV spectrum suggested the presence of quercetin) was collected and hydrolysed to yield quercetin and glucose. Therefore, owing to its relatively high polarity this peak was designated as quercetin-3-sophoroside, reported previously [14]. Concerning the peaks at 5.44 and 7.46 min, the first had the spectrum of a cinnamic acid derivative, whereas the second

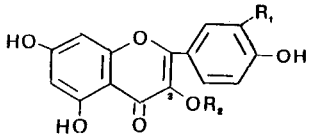
TABLE II

FLAVONES AND FLAVONOL GLYCOSIDES OF *ANTHEMIS NOBILIS* AND *EQUISETUM ARVENSE*



$R_1 = R_2 = OH$ Luteolin
$R_1 = OH$; $R_2 = H$ Apigenin

Species	Compound	No.
Anthemis nobilis	Apigenin-7-O-glucoside	IA
	Apigenin-7-O-apioglucoside	IIA
	Luteolin-7-O-glucoside	IIIA



$R_1 = OH$ Quercetin
$R_1 = H$ Kaempferol

Species	Compound	No.
Equisetum arvense	Isoquercitrin	IE
	Quercetin-3-O-sophoroside	IIE
	Astragalin	IIIE

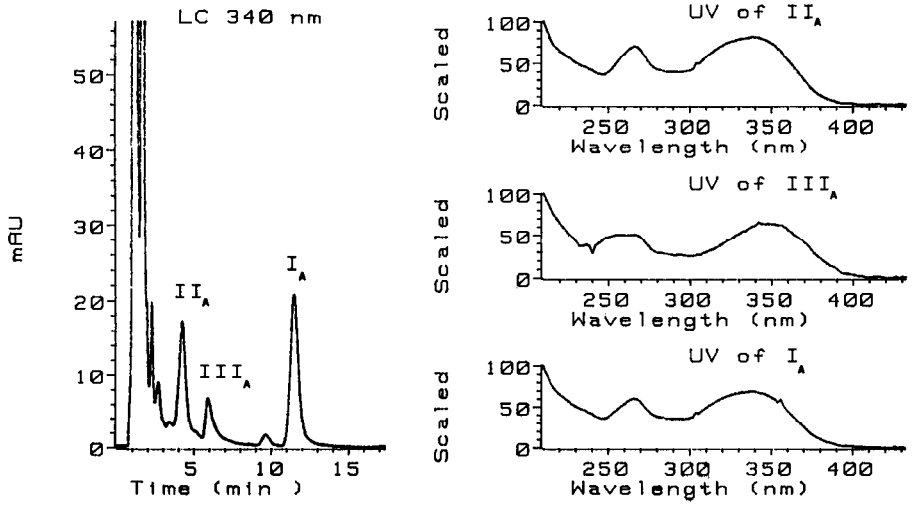


Fig. 5. Typical chromatogram from *Anthemis nobilis* flowers. Column, 7-μm C_8 Aquapore RP-300; eluent, 2-propanol–water (15:85); flow-rate, 2 ml/min. For peaks, see Table II.

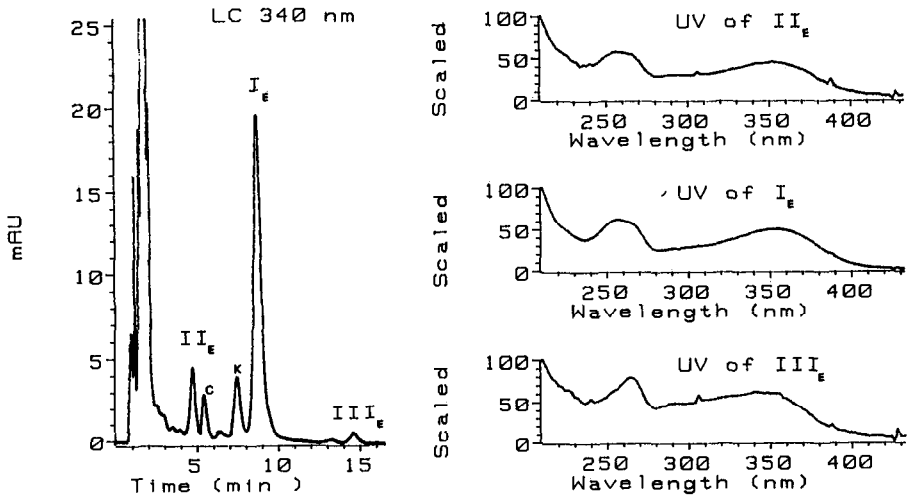


Fig. 6. Typical chromatogram from *Equisetum arvense* leaves. Column, 7-μm C_8 Aquapore RP-300; eluent, 2-propanol–water (15:85); flow-rate, 2 ml/min. For peaks, see Table II.

showed a spectrum typical of kaempferol glycosides (Fig. 7). Further investigation on these peaks was not carried out, as they eluted too closely and in very small amounts.

In conclusion, the combined use of a C_8 column and 2-propanol–tetrahydrofuran–water or 2-propanol–water systems was confirmed to be of great value for the sharp resolution of flavonoids from medicinal plants. Moreover, spectral data obtained with diode-array detection together with the results from the hydrolytic procedures and the retention times allowed the peak to be identified and their purities to be checked.

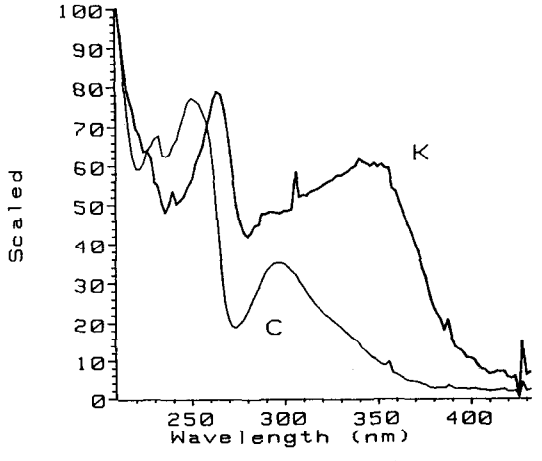


Fig. 7. UV spectra of peaks K and C in Fig. 6.

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REFERENCES

1 K. R. Markham, in J. B. Harborne (Editor), *Methods in Plant Biochemistry, Vol. 1, Plant Phenolics*, Academic Press, London, 1989, p. 208.
2 J. Daigle and E. J. Conkerton, *J. Liq. Chromatogr.*, 11 (1988) 309.
3 P. G. Petta, P. L. Mauri, E. Manera, P. L. Ceva and A. Rava, *Chromatographia*, 27 (1989) 509.
4 P. G. Pietta, P. L. Mauri, E. Manera and P. L. Ceva, *Chromatographia*, 28 (1989), 311.
5 P. G. Pietta, P. L. Mauri, E. Manera and P. L. Ceva, *J. Chromatogr.*, 513 (1990) 397.
6 P. G. Pietta, P. L. Mauri, C. Gardana, R. Mattei Facino and M. Carini, *J. Chromatogr.*, 537 (1991) 449.
7 P. G. Pietta, P. L. Mauri and A. Rava, *J. Chromatogr.*, 437 (1988) 453.
8 K. R. Markham, in J. B. Harborne (Editor), *Methods in Plant Biochemistry, Vol. 1, Plant Phenolics*, Academic Press, London, 1989, p. 229.
9 P. Albersheim, D. J. Nevins, P. D. English and A. Karr, *Carbohydr. Res.*, 5 (1967) 340.
10 C. Victoire, M. Haag-Berrurier, A. Lobstein-Guth, J. P. Balz and R. Anton, *Planta Med.*, 54 (1988) 245.
11 M. Vanhaelen and R. Vanhaelen-Fastre, *J. Liq. Chromatogr.*, 11 (1988) 2969.
12 M. Vanhaelen and R. Vanhaelen-Fastrè, *Planta Med.*, 55 (1989) 202.
13 H. Wagner, S. Bladt and E. M. Zgamski, *Plant Drug Analysis*, Springer, Berlin, 1984, p. 182.
14 M. Veit, F. C. Czygan, B. Frank, D. Hofmann and B. Worlicek, *Dtsch. Apoth.-Ztg.*, 129 (1989) 1591.