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DUAL-WAVELENGTH ABSORBANCE RATIO AND SPECTRUM SCANNING TECHNIQUES FOR IDENTIFICATION OF FLAVONOIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The use of absorbance ratio and UV–VIS spectrum scanning techniques is described for the identification and purity check of flavonoid compounds separated by high-performance liquid chromatography (HPLC). The possibility to correlate absorbance ratios, UV spectra and retention times with those of authentic samples greatly enhances the potential of HPLC for qualitative analysis. The techniques were used for the analysis of the major flavonoid compounds found in the plant *Uncaria elliptica*, namely rutin and (−)-epicatechin. The tissue distribution and the effect of reduced sunlight on the level of these flavonoids were studied. Limitations of our analytical techniques are also discussed.

INTRODUCTION

The use of absorbance ratio and spectrum scanning techniques for the identification and purity check of peaks obtained from high-performance liquid chromatography (HPLC) is gaining popularity. This is evident in the many recent analytical applications of these techniques for compounds such as peptides[1], ketones and aldehydes[2], barbiturates[3], zeranol[4], ascorbic and dehydroascorbic acids[5], alkaloids[6], serum constituents[7], drugs[8] and carotenoids[9]. However, few reports are available describing the use of these techniques for the analysis of flavonoid compounds[10].

Flavonoids are benzo-γ-pyrone derivatives which are found ubiquitously in plants. They are known to possess many pharmacological properties[11]. Ultraviolet spectroscopy has long been a major technique for flavonoid structure elucidation[12]. UV absorbance techniques are particularly attractive for flavonoid analysis. Using a programmable multiwavelength UV–VIS detector, we were able to demonstrate the discriminating capability and suitability of UV spectroscopy (Table I) for several flavonoid standards (Fig. 1).

The plant *Uncaria elliptica* is a woody climber, widely distributed in tropical regions and it is used as folk medicine and as a source of tannins[13,14]. Our earlier report[15] on the plant as a major source of rutin (quercetin-3-rutinoside) has aroused

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TABLE I

RETENTION TIME, CAPACITY FACTORS AND ABSORBANCE RATIOS OF FLAVONOIDS

Separation was carried out using HPLC system I (see text). Detector conditions: 0.05 a.u.f.s., ratio min. = 0.01, ratio max. = 100.00, and threshold = 5% f.s.

Flavonoids	*Retention time (min)*	*Capacity factor (k')*	*Absorbance ratios*			
			A_{260}/A_{290}	A_{280}/A_{290}	A_{330}/A_{290}	A_{360}/A_{290}
(1) (+)-Catechin	5.19	2.24	1.24	3.23	–	–
(2) (–)-Epicatechin	8.56	4.35	1.29	3.31	–	–
(3) Taxifolin	11.82	6.39	0.18	0.84	0.17	–
(4) Rutin	13.03	7.14	2.21	0.85	1.52	1.90
(5) Quercitrin	14.55	8.09	2.43	0.87	1.56	1.61
(6) Myricitrin	15.29	8.56	2.38	0.96	1.49	2.75
(7) Silybin	18.95	10.84	0.22	0.93	0.70	–
(8) 7-Hydroxyflavone	21.69	12.56	0.66	0.72	0.73	–
(9) 6-Hydroxyflavone	22.71	13.20	1.41	1.04	0.59	0.17
(10) Flavone	26.98	15.86	0.53	0.68	0.23	–

interest in the pharmaceutical industry. Analysis of the flavonoids of the plant have been limited to the use of paper chromatography elution-cum-colorimetry measurements. In this study, we have developed a simple, rapid and precise HPLC method to quantitate the major flavonoids of interest. As impurities can lead to erroneous quantitation, absorbance ratio and stop-flow scanning techniques were used for checking peak purity as well as for peak identification. We were also able to determine the tissue distribution of the two major flavonoids namely rutin and (–)-epicatechin in the *Uncaria elliptica* plant. The levels of these two flavonoids in plants grown in the shade were also studied.

EXPERIMENTAL

Sample

The plant materials were collected, prepared and extracted as described earlier[15].

Reagents and chemicals

HPLC-grade acetonitrile and tetrahydrofuran (THF) and analytical grade acetic acid were obtained from Merck (Darmstadt, F.R.G.). Pure water was obtained by de-ionising distilled water using a Barnstead NANOpure II system (Sybron/Barnstead, Boston, MA, U.S.A.). Flavonoid standards were purchased from Sarsyntex (Merignac, France).

Apparatus

A Waters HPLC system equipped with a Waters 840 data control station (combining a Digital professional 350 bench-top computer and Waters expert chromatography software), a system interphase module, two Model 510 pumps, a WISP au-

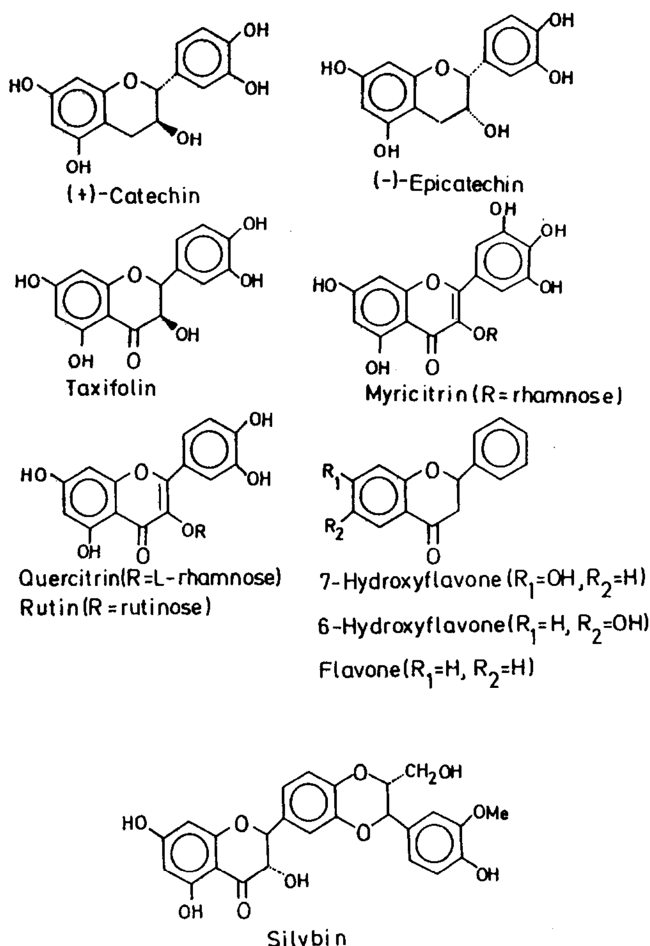

Fig. 1. Molecular structure of flavonoids.

tosampler, a SE 120 plotter and a Waters 490 programmable multiwavelength UV detector were used. We also used a Watford Control A.C. voltage stabilizer (Watford Control, Watford, U.K.) to protect the system against power fluctuations.

Column

The column used was a 15 cm × 4.0 mm I.D. MicroPak MCH-5-n-capp reversed-phase C_{18} column with 5-μm packing (Varian, Walnut Creek, U.S.A.).

Chromatographic procedures

The flow-rate was 1 ml/min. We have developed two elution programs for the separation of flavonoids.

System I. Solvent A: acetonitrile–water–acetic acid (8:89.5:2.5, v/v/v); solvent B: acetonitrile. Elution profile: 0–5 min: 100% A (isocratic); 5–20 min: 0–30% B in A (linear gradient); 20–37 min: 30–70% B in A (linear gradient); 37–40 min: 70% B in A (isocratic).

System II. Solvent A: acetonitrile–water (6:94, v/v); solvent B: acetonitrile–tetrahydrofuran–acetic acid–water (45:2.5:2.5:50, v/v/v). Elution profile: 0–9 min: 100% A; 9–21 min: 10% B in A (isocratic); 21–46 min: 10–30% B in A (linear gradient); 46–50 min: 30% B in A (isocratic).

The absorbance ratios were determined for $A_{260\text{ nm}}/A_{290\text{ nm}}$, $A_{280\text{ nm}}/A_{290\text{ nm}}$, $A_{330\text{ nm}}/A_{290\text{ nm}}$ and $A_{360\text{ nm}}/A_{290\text{ nm}}$ using the 4 analog channels of the detector. The spectra of the HPLC peaks were scanned from 190 to 500 nm using the stop-flow technique.

Quantitation

Standard curves of peak height against concentration of rutin and (−)-epicatechin (in the range 0–5 μg) were obtained using the computer software (multi-method least square fit calibration mode). The detection was carried out at 280 nm, with the detector sensitivity set at 0.1 a.u.f.s. and a threshold at 5% f.s. The mobile phase used was as described for system II. During each extraction of the plant tissues, quercitrin was used as an internal standard and added (10 mg/g dried tissue) prior to homogenization in order to ascertain the percentage of recovery.

RESULTS AND DISCUSSION

Our results showed that the use of several absorbance ratios at suitable wavelengths could distinguish the flavonoids (Table I). The ratios obtained were characteristic of each flavonoid in a given mobile phase, regardless of solute concentration[16], column efficiency, lamp energy, peak shape, flow-rate and retention time. Any impurities that co-eluted or overlapped with the peak of interest would be indicated by a distortion and alteration of both the spectrum and ratiogram.

The advocated use of several absorbance ratios[17] is important in the determination of peak purity and identity. A single ratio is often insufficient to index peak homogeneity. This was illustrated in Fig. 2b where the ratioplot at $A_{280\text{ nm}}/A_{290\text{ nm}}$ appeared to indicate peak homogeneity. However, the use of additional ratioplots at $A_{260\text{ nm}}/A_{290\text{ nm}}$ and $A_{360\text{ nm}}/A_{290\text{ nm}}$ (Fig. 2c and d) indicated that peak 6 (myricitrin) had some contaminations as shown by the distortion to the "flat-topped" or "block-shaped" peaks. Peaks 1 (catechin), 2 (epicatechin), 3 (taxifolin), 7 (silybin), 8 (7-hydroxyflavone) and 10 (flavone) were absent in Fig. 2d because at the ratioplot used (360 nm/290 nm) the absorbance did not exceed the threshold value of 5% f.s.

We propose here the use of 290 nm as the master (denominator) wavelength for the absorbance ratio analysis of flavonoids. This wavelength lies in between the absorption band I and II[12] of flavonoids and has minimal absorptivity for most if not all of the flavonoids. The operating (numerator) wavelength for the absorbance ratio would be best selected to lie either within the band I or band II region where the UV absorptivity of the flavonoids is maximal, such that the resulting ratio (*i.e.* operating wavelength/master wavelength) would exhibit a larger value.

The dependence of the absorbance ratios and UV–VIS spectra on the mobile phase has been considered to be a major practical limitation of the technique[18]. However, we feel that this drawback may be turned into an advantage, because upon changing the mobile phase new ratio values are obtained, which give additional information on the purity and identity of the compounds under investigation.

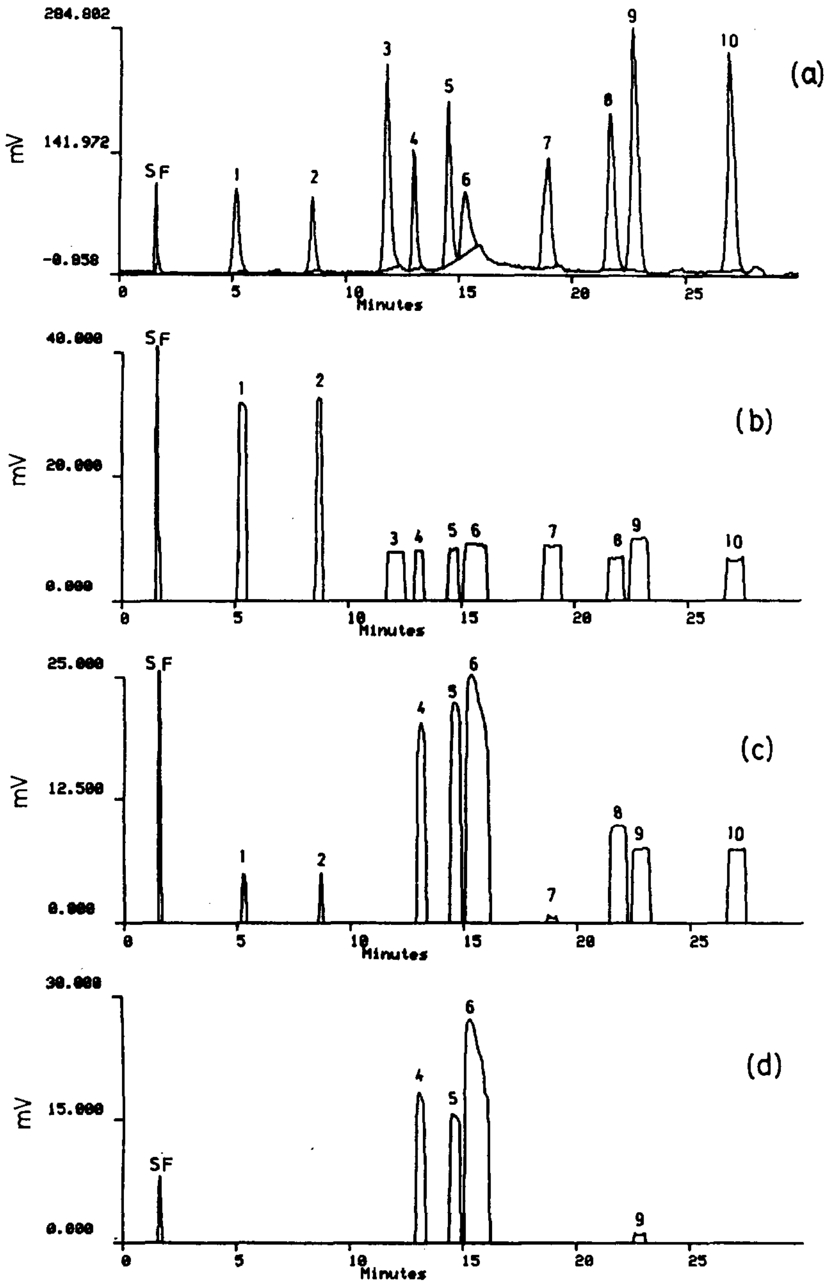

Fig. 2. High-performance liquid chromatograms of the flavonoids (+)-catechin (1), (−)-epicatechin (2), taxifolin (3), rutin (4), quercitrin (5), myricitrin (6), silybin (7), 7-Hydroxyflavone (8), 6-hydroxyflavone (9) and flavone (10). Separation was carried out using HPLC system I (see text). Detector conditions: (a) Maxplot (maximum absorbance) at 254, 280 and 360 nm simultaneously, 0.1 a.u.f.s., (b) ratioplot, 280 nm/290 nm, (c) ratioplot, 260 nm/290 nm, (d) ratioplot, 360 nm/290 nm; ratio min = 0.01, ratio max = 100 00, 0.1 a.u.f.s., and threshold = 5% f.s.

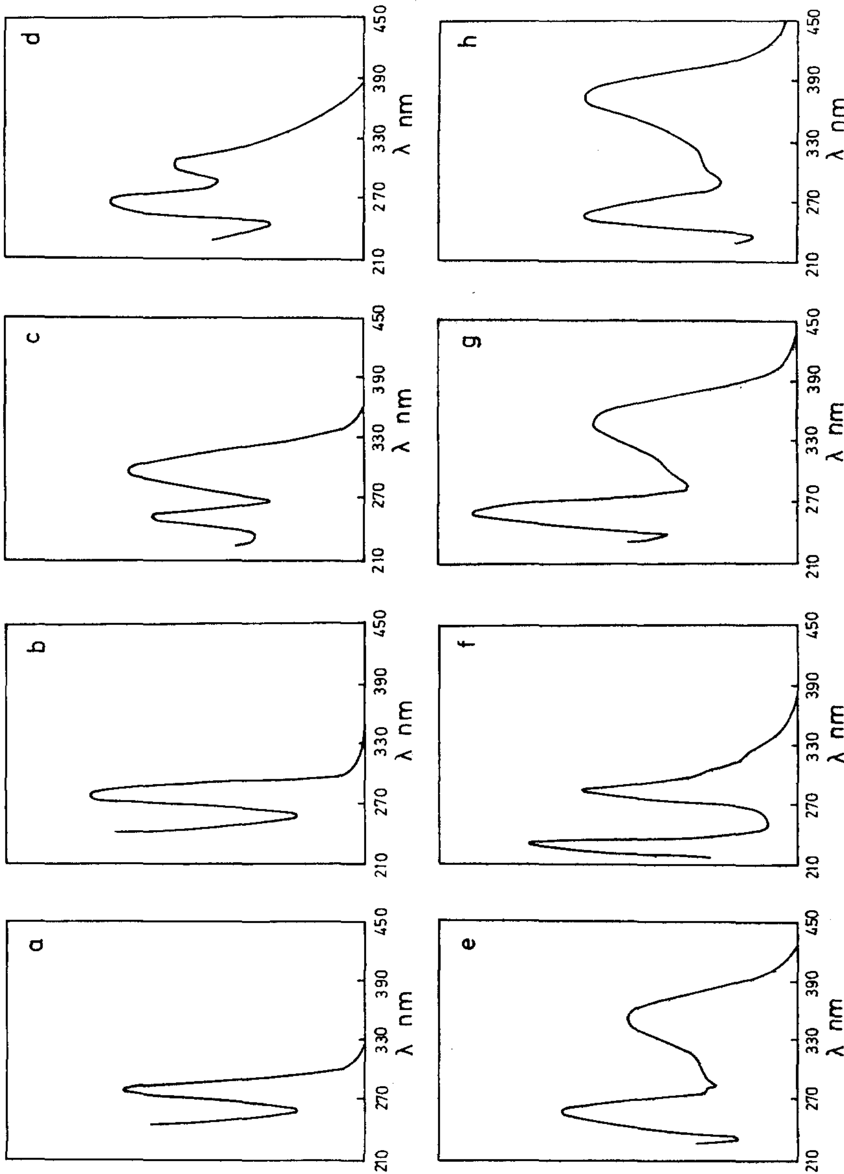

Fig. 3. UV spectra of flavonoid standards separated by HPLC system I (see text), using the stop-flow scanning technique. Wavelength step = 1 nm, step period = 0.1 s, scan a.u.f.s. = 0.1, chart speed = 10 cm/min, auto range = yes, tic marks = off. (−)-Epicatechin (a), (+)-catechin (b), flavone (c), 6-hydroxy flavone (d), rutin (e), taxifolin (f), quercitrin (g), myricitrin (h).

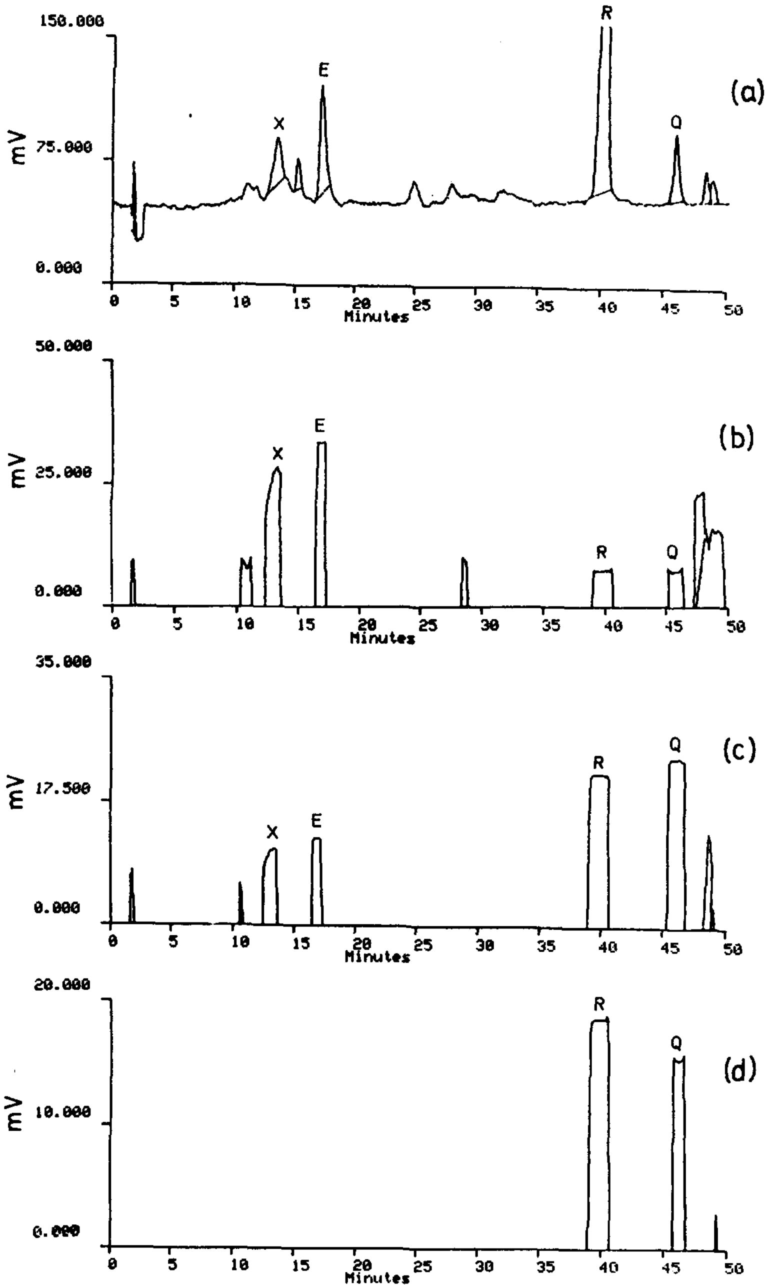

Fig. 4. High-performance liquid chromatograms of a crude methanolic extract of *Uncaria elliptica* using HPLC system II (see text). Detector conditions: as in Fig. 2. (−)-Epicatechin (E), rutin (R), quercitrin (Q), unknown (X).

The major drawback of the UV–VIS spectra obtained by the stop-flow scanning technique is the lack of fine structures (Fig. 3). Based on UV–VIS spectra alone, it is difficult or impossible to differentiate between two compounds having similar chromophores, as in the case of the diastereoisomers (−)-epicatechin and (+)-catechin (Fig. 3a and b). However, we have found that the absorbance ratios allow a certain discrimination between the two flavonoids (Table I). Hostettmann *et al.*[10] reported the use of a post-column spectrum shifting technique for flavonoid identification, but we have found that the combination of absorbance ratios and UV–VIS spectra gives a simplified and improved method for peak identification. Furthermore, compounds with similar chromophores exhibit closely related absorbance ratios and spectra. This feature could be used for the tentative classification of unknown peaks. Thus, for example, the unknown peak X in Fig. 4 most probably belongs to the flavan-3-ol family owing to its close absorbance behaviour to (+)-catechin and (−)-epicatechin. Studies are being carried out to identify this peak.

TABLE II

RUTIN AND (−)-EPICATECHIN CONTENTS IN LEAVES AND WOODS OF DIFFERENT AGES OF THE PLANT *UNCARIA ELLIPTICA* BY HPLC DETERMINATION

Separation was carried out using HPLC system II (see text). Detector conditions: 280 nm, 0.1 a.u.f.s., and threshold = 5% f.s. Content is given in % (w/w) for dry tissue. Mean values and standard deviations are calculated from three measurements.

Position and state of plant tissues	*Rutin content*	*(−)-Epicatechin content*
Apical and below apical, young leaves	25.6 ± 0.3	0.9 ± 0.1
	16.8 ± 0.5*	1.5 ± 0.3*
Intermediate, mature leaves	21.0 ± 0.6	2.0 ± 0.1
	11.9 ± 0.2*	2.9 ± 0.2*
Basal, old leaves	11.8 ± 0.8	4.0 ± 0.3
	13.8 ± 1.1*	3.8 ± 0.1*
Apical and below apical, young twigs	11.3 ± 0.4	1.2 ± 0.1
	7.5 ± 0.2*	3.3 ± 0.2*
Intermediate, mature woods	2.7 ± 0.4	1.1 ± 0.3
	2.8 ± 0.3*	1.3 ± 0.1*
(a) Bark	6.9 ± 0.1	1.5 ± 0.03
	4.7 ± 0.4*	3.0 ± 0.1*
(b) "woody" tissue	0.6 ± 0.03	0.5 ± 0.04
	0.6 ± 0.1*	0.4 ± 0.1*
(c) "central" pith	1.9 ± 0.04	0.6 ± 0.1
	0.04 ± 0.05*	0.5 ± 0.1*
Basal, old woods	1.0 ± 0.08	0.9 ± 0.07
	2.5 ± 0.3*	1.1 ± 0.1*
(a) Nark	2.7 ± 0.1	2.2 ± 0.06
	2.5 ± 0.3*	1.6 ± 0.2*
(b) "woody" tissue	0.5 ± 0.05	0.4 ± 0.03
	0.7 ± 0.2*	0.3 ± 0.1*
(c) "central" pith	1.5 ± 0.1	0.4 ± 0.03
	1.5 ± 0.1*	0.4 ± 0.1*

* Plants grown in the shade.

The HPLC separation of a crude extract of *U. elliptica* and some of the corresponding ratioplots are shown in Fig. 4. The best results were obtained with gradient elution since the compounds have relatively large differences in their capacity factors. The gradient run in system II involved a pH gradient (or pH shift) from pH 7 to pH 2.8. This was to remove any interference from phenolic acids[19]. The addition of acetic acid and tetrahydrofuran to the mobile phase improved the peak shape and the resolution of later eluting peaks.

The HPLC method described here for the plant extract is more rapid, more sensitive, and more precise when compared to the paper chromatography method[15]. The percentage of recovery from our extraction method for the flavonoids using quercitrin as the internal standard was in the range 72–80%. The amount of rutin determined by the HPLC method gave higher values while that of (−)-epicatechin showed lower values. The pattern of the distribution of rutin and (−)-epicatechin over the leaves and woods of different ages was similar to that reported earlier[15]. The young leaves, young twigs and slightly more mature leaves of plants growing in the shade were found to have a lower rutin but a moderately higher (−)-epicatechin content, indicating that sunlight affects the metabolism and/or the translocation of these flavonoids. In addition, we have found that rutin, (−)-epicatechin and other UV absorbing compounds were present in relatively higher concentrations in the bark than in the pith and in the woody tissue of the stem (Table II). This distribution pattern is probably related to the possible role of flavonoids as a sun screen and in the defence against toxins and pathogens[20].

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