HPTLC and reflectance mode densitometry of anthocyanins in *Malva Silvestris L.*: a comparison with gradient-elution reversed-phase HPLC [☆]

A. Farina [a,*], A. Doldo [a], V. Cotichini [a], M. Rajevic [a], M.G. Quaglia [b], N. Mulinacci [c], F.F. Vincieri [c]

[a] *Laboratorio di Chimica del Farmaco, Istituto Superiore di Sanità, V. le Regina Elena 299, 00161 Rome, Italy*
[b] *Dipartimento Studi Farmaceutici, Università "La Sapienza", P. le A. Moro 5, 00185 Rome, Italy*
[c] *Dipartimento di Scienze Farmaceutiche, Università di Firenze, via G. Capponi 9, 50121 Florence, Italy*

Abstract

Aqueous alcoholic mallow flower extracts were analyzed both by HPTLC–densitometry in the reflectance mode at 530 nm and by reversed-phase HPLC with gradient elution. For the mallow flower anthocyanins the best chromatographic resolution was obtained by HPLC, which revealed only two main compounds, confirmed by FAB-MS: malvidin 3,5-O-diglucoside (malvin) and malvidin 3-O-(6″-O-malonylglucoside)-5-O-glucoside. The HPTLC densitometric method on cellulose plates provides accuracy, reproducibility and selectivity for the quantitative analysis of the anthocyanins and this method was shown to be much more sensitive than the HPLC-DAD system, at 530 nm. Both methods give comparable quantitative results for total anthocyanins when applied to mallow flowers from two different sources: Italy and Albania.

Keywords: Mallow; Anthocyanins; Malvin; HPTLC; Gradient-elution HPLC; Reversed-phase separations; Reflectance mode densitometry

1. Introduction

Aqueous alcoholic extracts of flowers and leaves of *Malva Silvestris L.* (mallow) have been widely used as a mild relief for cough and inflammatory diseases of the mucous membrane.

Polysaccharides, flavonoids and anthocyanins are the main active components of the herb. Chemically, the anthocyanins are salts of glycosylated derivatives of the 3,5,7,4′-tetrahydroxyflavilium cation. Glycosylation occurs at the 3,5 and 7 positions. Since the aglycone anthocyanidins are unstable in water and much less soluble than anthocyanins, glycosylation is assumed to confer solubility and stability to the pigment. In many cases the sugar residues are acylated and the acyl

* Corresponding author.

substituents are usually bonded to the C-6″ position of the sugar moiety at C-3 in the anthocyanin [1,2]. All these compounds readily degrade under the action of various agents such as light and oxygen, making their evaluation difficult.

Malvidin 3,5-O-diglucoside (I; malvin), malvidin 3-O-glucoside (II; oenin), delphinidin 3-O-glucoside [3] (IVₐ) and also the 3-O-(6″-O-malonylglucoside) of malvin [4] (V) have been perviously identified as derivatives of malvidin (III) or delphinidin (IV) respectively, in mallow flowers (Fig. 1).

Anthocyanin identification has been carried out for many years by means of paper chromatography (PC); more recently HPLC, TLC and HPTLC have provided very good qualitative and quantitative means for the evaluation of these compounds [5]. For mallow flowers the separation of anthocyanins has been carried out by TLC on silica gel and cellulose layers using various eluents [6].

The aim of the present study on the aqueous alcoholic extracts of mallow flowers was: (a) to develop an HPTLC densitometric procedure for the rapid identification and quantitation of anthocyanins; and (b) to evaluate the combination of HPTLC and HPLC for the qualitative and quantitative characterisation of the anthocyanins.

2. Experimental

2.1. Materials

Reference standards of the chloride salts of III, IV and II were obtained from Extrasynthèses (Genay, France). I was obtained from Fluka (Ref. 63440; Buchs, Switzerland).

Mallow flower samples were kindly supplied by D. Ulrich, Turin, Italy (collected in 1994 in Albania) and by Aboca s.r.l. (Arezzo, Italy).

2.2. Procedure

2.2.1. Standard solutions

Standard solutions in acidified methanol (0.5% v/v HCl) of each reference standard (100 ng µl⁻¹) were prepared immediately before

use, for determination of the R_f values and, after appropriate dilution (1:10 or 1:100), for quantitative purposes. The stock solution were stored at 0 °C under nitrogen in the dark, and freshly prepared every week.

2.2.2. Extraction of anthocyanins

10 g of crushed flowers or petals were extracted for ≈20 h at room temperature in the dark and under nitrogen using 200 ml of 20% v/v ethanol containing citric acid (0.15% w/v) and acidified to pH* 2.5 with 6 M HCl. The mixture was filtered and the residue washed with the same solvent until a colourless filtrate was obtained. For every extract prepared the final drug:solvent ratio range from 1:20 to 1:50 w/v. The final extract (pH* 4.2) was acidified to pH* 2.5 and 6 M HCl.

2.2.3. Clean-up of crude extract

The crude extract of anthocyanins was concentrated under vacuum to yield an aqueous residue and applied to a 3 ml disposable Extrelut® car-
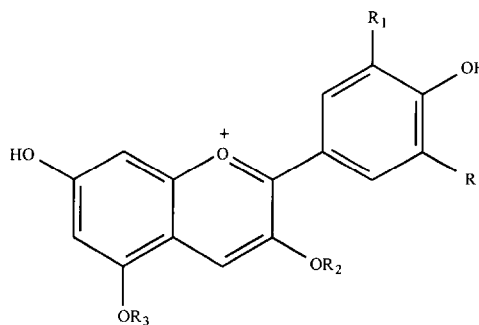
		R_1	R_2	R_3
I	Malvidin 3,5-O-diglucoside (malvin)	OCH₃	Glu	Glu
II	Malvidin 3-O-glucoside (oenin)	OCH₃	Glu	H
III	Malvidin	OCH₃	H	H
IV.	Delphinidin 3-O-glucoside	OH	Glu	H
V	Malvidin 3-O-(6″-O-malonylglucoside) 5-O-glucoside	OCH₃	Glu-6″-O-malonyl	Glu

Fig. 1. Structural formulae of the anthocyanins in the mallow flowers.

tridge (Merck, Darmstadt, Germany). Elution was performed first with hexane, then with ethyl acetate and finally with methanol. The methanolic extract of anthocyanins was acidified to pH* 2.5 with 6 M HCl.

2.2.4. Purified anthocyanin fraction for FAB-MS

The crude extract of dried petals was concentrated under vacuum to yield an aqueous residue and applied to a 20 ml disposable Extrelut® cartridge. The first elution was performed with ethyl acetate and the second with methanol acidified with formic acid (24% v/v; pH* 2.5). The methanolic fraction was dried under vacuum, redissolved in acidified water (formic acid, pH 2.5) and applied to a BondElut C_{18} cartridge (Analytichem International). Elution was performed with ethyl acetate and then with acidified methanol (formic acid, 2.4% v/v, pH* 2.5).

FAB-MS analysis was performed on this methanolic extract which contained only the anthocyanins. The mass spectra were obtained on a VG ZAB2F instrument (VG Division of Fisons, Manchester, UK), under positive FAB conditions: 8 keV Xe atoms bombarding a glycerol solution of the sample.

2.2.5. Acid hydrolysis

Acid hydrolysis was performed on 2 ml of the extract which was diluted with 1 ml of 6 M HCl and heated for 1 h at 100 °C over a boiling water bath.

2.2.6. Alkaline hydrolysis

5 ml of the aqueous alcoholic extract was added to 5 ml of 10% w/v KOH under nitrogen; after 20 min at room temperature, the mixture was acidified with concentrated HCl.

2.2.7. HPTLC densitometry

Chromatography was performed on analytical HPTLC plates (10 cm × 20 cm) precoated with (a) cellulose of 0.1 mm thickness, (b) Silica Gel 60 of 0.2 mm thickness (Merck, Darmstadt, Germany). Samples (1–10 μl) were applied to the plates as 10 mm bands (8 mm from the lower edge of the plate) by means of a Linomat IV applicator (Camag, Muttenz, Switzerland) (rate of delivery

6 s μl^{-1}; 13 bands per plate). Plates were developed for about 5 cm from the baseline (development time: about 30 min) in saturated horizontal developing chambers (Camag, Muttenz, Switzerland) with formic acid–5.5% hydrochloric acid (2:1, v/v) for cellulose plates, and with ethyl acetate–butanone–formic acid–water (5:3:1:1, v/v/v/v) for silica gel plates. Developed plates were dried with a stream of cold air and quantified by linear scanning in the reflectance mode at 530 nm, by means of a Camag HPTLC-Scanner (Muttenz, Switerzland) that was computer aided (CATS3 software residing on an Olidata 433 computer with a 80486 processor). Chromatograms of reference anthocyanins were obtained by spotting standard solutions in acidified methanol (0.5% v/v HCl). The analysis conditions were: band width, 0.4 mm; band length, 4 mm; slit, 10 nm; scan speed, 2.0 mm s^{-1}.

2.2.8. HPLC

The extracts were filtered through Millex-HA 0.45 μm (Millipore) filters and analysed by HPLC on the following modular system: a gradient pump system (Perkin-Elmer series 200; Norwalk, CT, USA) with a diode-array detector (Waters, Model DAD 994; Milford, MA, USA) at 530 nm (band width 8 nm) and a loop-valve injector (20 μl; Rheodyne, Model 7161, Cotati, CA, USA) under the following conditions: guard column: Supelguard LC18, 2 cm (Supelco); column: Nucleosil RP18 300 Å, 5 μm (250 mm × 4 mm) (S.G.E.). The solvents were (A) H_2O–HCOOH–CH_3COOH (1000:7:9, v/v/v; pH 2.2); and (B) acetonitrile. Gradient profile: 0–20 min 10–90% B in A (linear gradient). Flow rate: 0.75 ml min^{-1} at ambient temperature. The retention times were: **I** 8.3 min; **V** 9.2 min; **II** 9.8 min; **IV** 10.4 min; **III** 12.4 min.

3. Results and discussion

Densitometric reflectance scanning profiles of HPTLC chromatograms at 530 nm for **I**, **II**, **III** and **IV** standards on (a) cellulose and (b) silica gel are shown in Fig. 2. Good resolution and a good spread of R_f values are observed in both cases, but
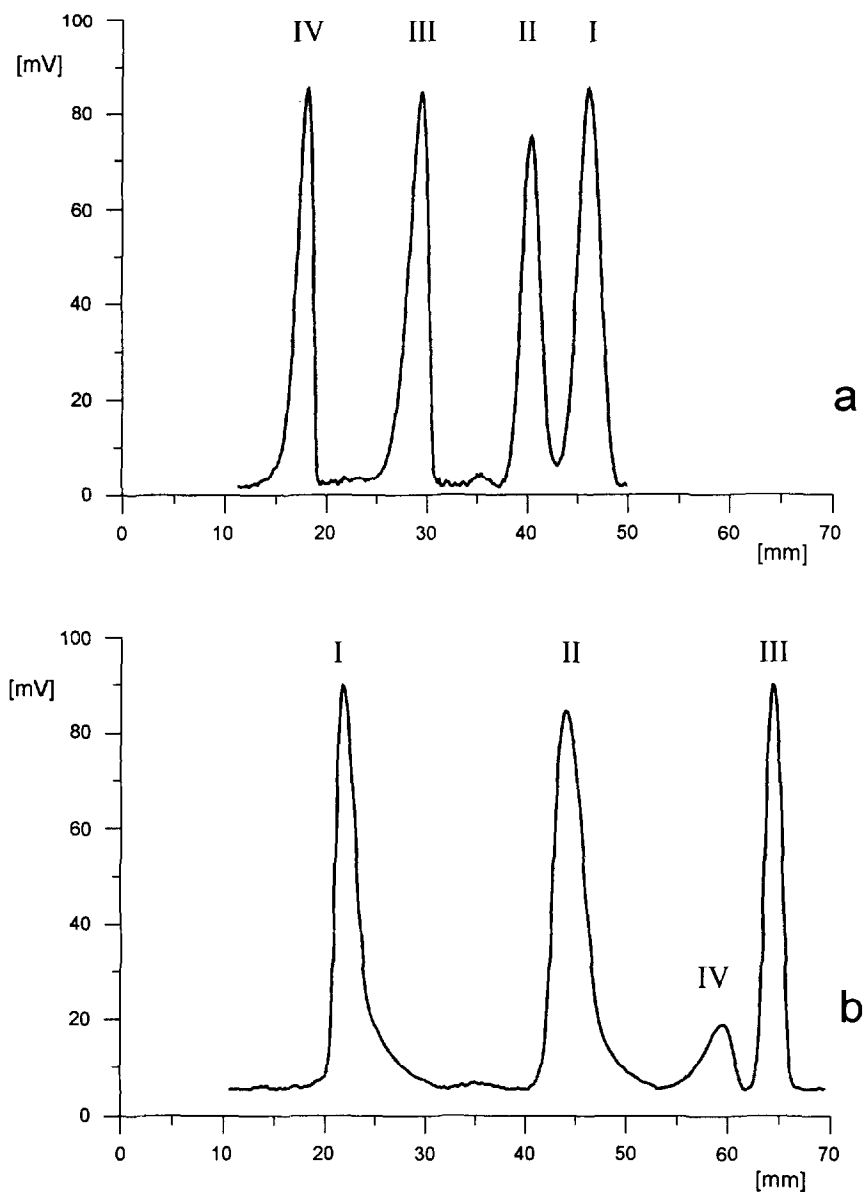
Fig. 2. Densitometric reflectance mode scanning profile of HPTLC chromatograms of the standard mixture at 530 nm on (a) cellulose; (b) silica gel. The concentrations of **I**, **II**, **III** and **IV** were 10 ng μl^{-1} each in acidified methanol (0.5% v/v HCl). The acidic liquid phases used were formic acid–5.5% v/v hydrochloric acid (2:1, v/v) for cellulose; and ethyl acetate–butanone–formic acid–water (5:3:1:1, v/v/v/v) for silica gel.

for anthocyanidins silica plates (and the respective liquid phase) are not completely suitable for quantitative analysis since the spots fade rapidly, especially that of **III**.

Linearity, repeatability, and detection limit data for these compounds are presented in Table 1. The lowest limits for practical quantitation purposes were approximately three times the LOD.

Table 1
Comparison of linearity, repeatability and detectability of HPTLC reflectance data for anthocyanins and anthocyanidins on (a) cellulose and (b) silica gel plates with detection at 530 nm

Parameter	I		II		III		IV	
	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
Linear range [a]	15–100 ($n = 10$)	35–250 ($n = 10$)	15–100 ($n = 10$)	100–800 ($n = 10$)	15–100 ($n = 10$)	135–1000 ($n = 10$)	15–100 ($n = 10$)	n.m. [c]
Correlation coefficient	0.9958	0.9915	0.9963	0.9930	0.9980	0.9974	0.9968	n.m. [c]
RSD (%)	5.5 at 50 ng ($n = 8$)	1.3 at 100 ng ($n = 8$)	n.m. [c]	n.m. [c]	3.8 at 30 ng ($n = 8$)	n.m. [c]	n.m. [c]	n.m. [c]
Detection limit [b] (ng spot^{-1})	<5	30	5	50	5	n.m. [c]	5	n.m. [c]

[a] Concentration range, corresponding to nanograms applied to the plate.
[b] Signal-to-noise ratio = 2:1.
[c] n.m. = not measured.

A scanning profile of a separation by HPTLC on a cellulose plate obtained at 530 nm from mallow flower extract is presented in Fig. 3a. I is the main component identified, which is completely transformed to III after acid hydrolysis (Fig. 3a').

Scanning profiles of HPTLC chromatograms obtained using silica gel plates indicate that the peak corresponding to the diglucoside (I) corresponds to the overlapping of at least two components (Fig. 3b).

Better resolution was obtained by gradient-elution HPLC analysis on Nucleosil RP18. Fig. 4 shows HPLC profiles of mallow flower extract before and after alkaline (c) and acid (d) hydrolysis, compared with a standard mixture. Two well-resolved peaks were detected in the HPLC chromatogram of the mallow flower extract (b): one was due to I and the main component could resonably be suggested as being due to V, as previously reported in the fresh flowers of mallow [4]. This hypothesis is supported by the following considerations. First the UV–Vis spectra of I and V overlap (with λ_{max} values at 280 and 530 nm respectively). Moreover the derivatives of I, in reversed-phase HPLC, have been described as having higher t_R values with respect to the diglucoside I, as found here (Fig. 4a). The FAB-MS spectra, performed on this purified anthocyanin fraction revealed two intense molecular ions, at m/z 741 corresponding to V and at m/z 655 relating to I.

The HPLC profile of the mallow flower extract, after acid hydrolysis for 2 months at 4 °C, showed an increase in peak I and a corresponding decrease in peak V. This confirms the transformation of the malonyl derivative V into I in the presence of inorganic acids, as previously reported [1,2]. The identity of V was confirmed by HPLC comparison with the reference substance. Alkaline hydrolysis of the extract induced an increase in the peak area for I in HPLC, while peak V completely vanished. Acid hydrolysis of the extract induced a complete transformation of both I and V to yield malvidin (III), as can be seen in Fig. 4d.

Table 2 reports data relating to the quantitative determination of anthocyanins in mallow flower and petal extracts using both HPTLC (on cellulose plates) and HPLC. The HPTLC data represent total anthocyanins (calculated as I) and as such they agree well with the data found by HPLC.

In the mallow petal extract a fourfold higher concentration of the total anthocyanins was found, compared with the flower extract. HPLC
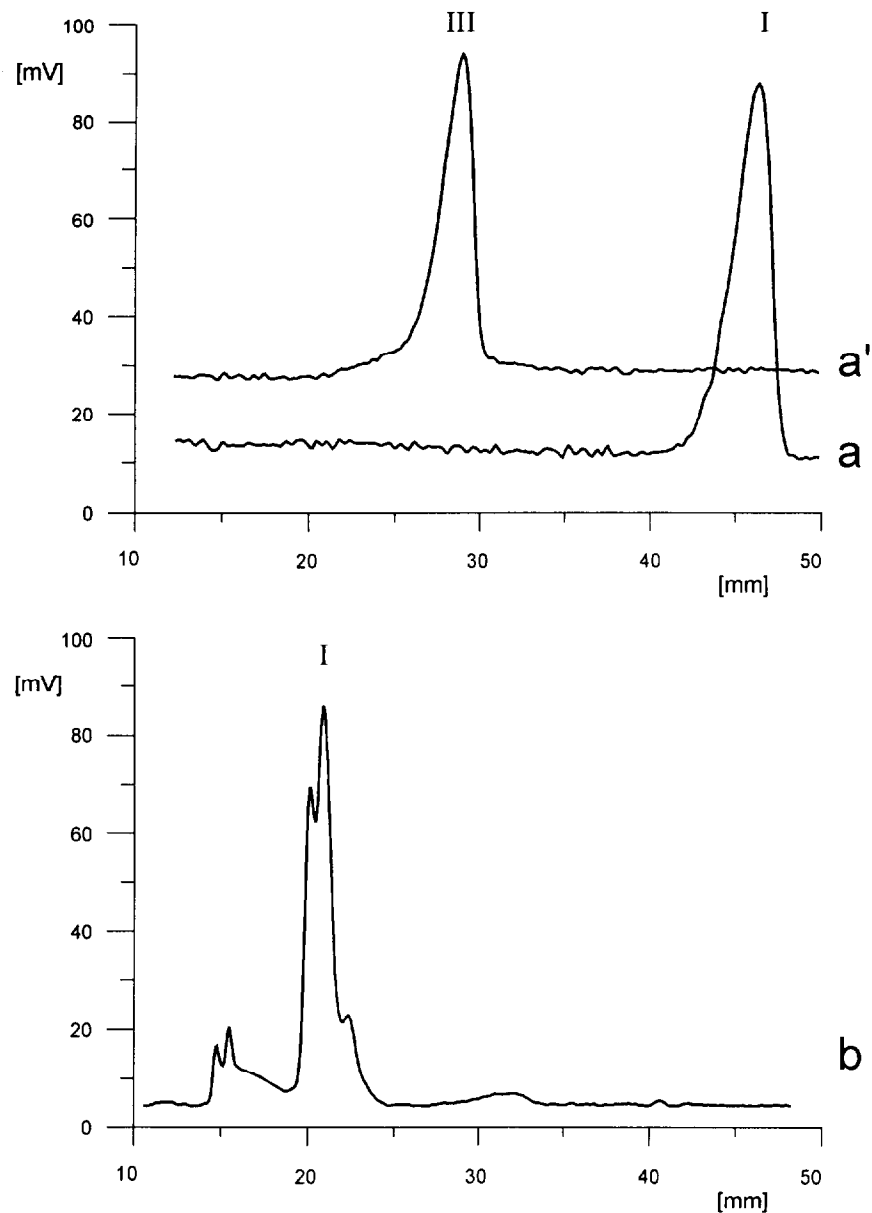
Fig. 3. Densitometric reflectance mode scanning profile of HPTLC chromatograms on (a, a') cellulose and (b) silica gel plates. (a, b) mallow flower extract; (a') mallow flower extract after acid hydrolysis. The acidic liquid phases used were formic acid–5.5% v/v hydrochloric acid (2:1, v/v) for cellulose; ethyl acetate–butanone–formic acid–water (5:3:1:1, v/v/v/v) for silica gel.

of the mallow petal extract shows a similar profile to that of the mallow flower extract, but with two additional minor components: one corresponding to II, while the other was unidentified (Fig. 5). Comparing the HPLC quantitative data for I and V in mallow petals from two different sources, the total content of anthocyanins was broadly similar, but the ratio of I and V was quite different in each case (1:2.5 for Albanian petals and 2:1 for petals of Italian origin). It should be noted that the content of V was calculated with reference to I, since their spectra were very similar.

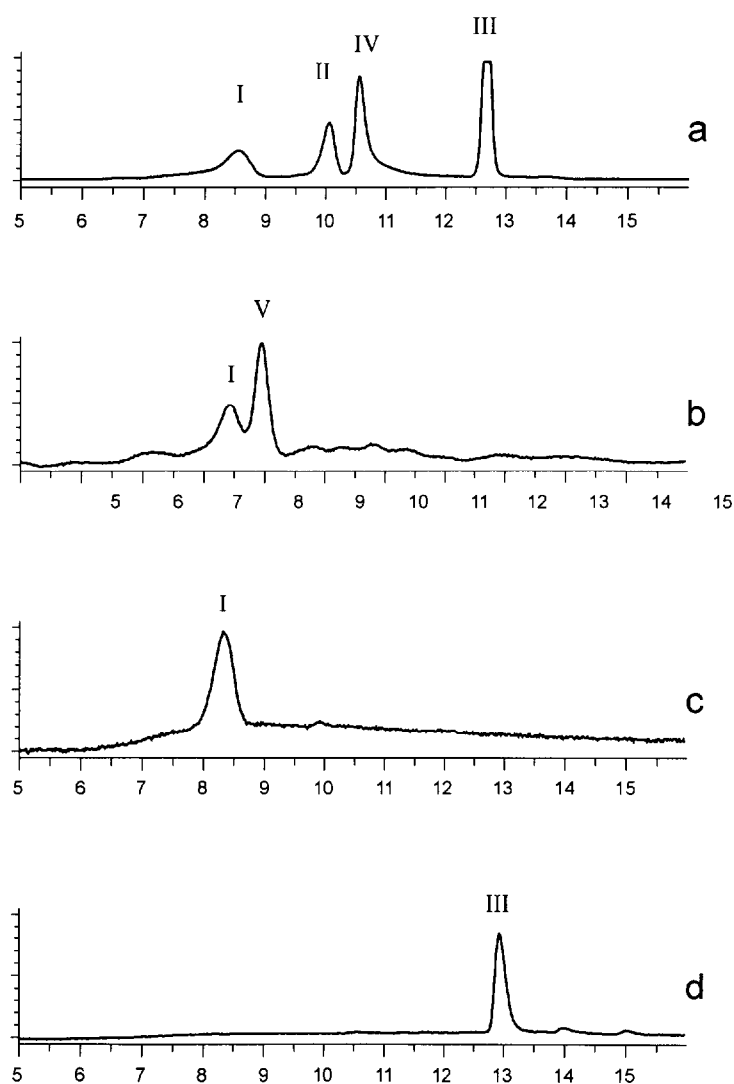Fig. 4. HPLC profiles (20 μl) at 530 nm on Nucleosil RP18 under gradient elution conditions (see text) of (a) mixture of standards each at 10 ng μl^{-1} in the solvent specified in Fig. 2; (b) mallow flower extract; (c) mallow flower extract after alkaline hydrolysis; (d) mallow flower extract after acid hydrolysis.

4. Conclusions

The proposed HPTLC densitometric method in reflectance mode provides accuracy, reproducibility and selectivity for the identification and determination of anthocyanin content in mallow flower and petal extracts. The anthocyanin content may be quantitated (a) as **I** directly in the extract or (b) as **III** after acid hydrolysis of the extract. Three duplicate samples and six standards (comprising three different concentrations in duplicate) can be separated and quantified within a few hours.

Better resolution was obtained by HPLC analysis compared to the HPTLC method, but the LOD of the HPLC-DAD system is substantially worse than that for HPTLC. No significant differences were observed in the quantitative results obtained by HPTLC and HPLC when applied to mallow flower and petal extracts without previous clean-up.

Table 2
Anthocyanin content of mallow flowers (g per 100 g flowers or petals; mean of three replicates except where stated)

Sample	HPTLC [a] on cellulose (total anthocyanin)	HPLC			
		Product I	Product V [a]	Product II	Total
Ulrich-mallow flowers [b]	0.12 ± 0.02 (n = 5)	0.036	0.073	n.d. [f]	0.109
Ulrich-mallow flowers [b] + 0.04% w/w of added malvin	0.155 [d]	0.069 [e]	0.073	n.d. [f]	0.142
Ulrich-mallow petals [b]	0.47	0.14	0.36	0.01	0.51
Aboca-mallow petals [c]	n.m. [g]	0.28	0.14	n.d. [f]	0.42

[a] Calculated with reference to I.
[b] Country of origin: Albania.
[c] Country of origin: Italy.
[d] Corresponding to about 85% of recovery of added I.
[e] Corresponding to about 83% of recovery of added I.
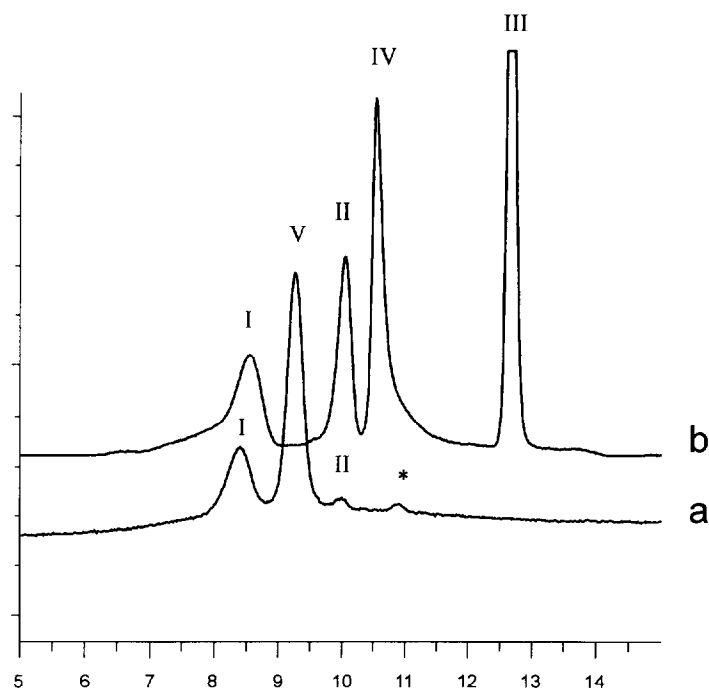[f] n.d. = none detected.
[g] n.m. = not measured.

Fig. 5. Comparison between HPLC profiles of (a) extract of mallow petals; (b) mixture of standards (as in Fig. 4). Asterisk indicates an unidentified compound. Experimental conditions as in Fig. 4.

The HPTLC densitometric method provides good resolution of the principal flavonoids extracted simultaneously with anthocyanins. The possibility of high sensitivity detection based on their intrinsic fluorescence forms the basis of further developments on the HPTLC determination of these compounds in the authors's laboratories.

References

[1] J.B. Harborne, Phytochemistry, 3 (1964) 151–160.

[2] J.B. Harborne, Bull. Liaison—Groupe Polyphenols, 15 (1990) 15–24.

[3] H. Pourrat, O. Texier and C. Barthomeuf, Pharm. Acta Helv., 65 (1990) 93–96.

[4] K. Takeda, S. Enoky, J.B. Harborne and J. Eageles, Phytochemistry, 28 (1989) 499–500.

[5] H.S. Lee and V. Hong, J. Chromatogr, 624 (1992) 221–234.

[6] H. Wagner, S. Baldt and E.M. Zkainski, in Plant Drug Analysis, Springer-Verlag, Berlin, 1984, pp. 270–273.