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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC CHARACTERIZATION OF SOME MEDICAL PLANT EXTRACTS USED IN COSMETIC FORMULAS

H. SCHULZ* and G. ALBROSCHEIT
DRAGOCO, Research Department, Analytical Laboratories, D-3450 Holzminden (F.R.G.)
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SUMMARY

Rapid and reliable methods are presented for the characterization of biologically active and/or characteristic constituents in aqueous extracts of *Hamamelis virginiana, Matricaria chamomilla, Achillea millefolium, Thymus vulgaris, Althaea officinalis* and *Cinchonia* spp. Prior to high-performance liquid chromatographic (HPLC) separation a clean-up step was performed using a solid-phase extraction system. The purified extracts were analysed by HPLC coupled with a diode-array detector and a fluorescence detector. In some instances, previously unreported components of the aqueous plant extracts were found.

INTRODUCTION

In recent years, the use of medical plant extracts in cosmetic products has increased considerably. Whereas in the past the characterization and standardization of these plant materials had been performed mostly by thin-layer chromatographic (TLC) or high-performance TLC methods[1–5], analysis by high-performance liquid chromatography (HPLC) has become increasingly convenient for this purpose. Most workers who have applied HPLC to plant ingredients have used C_{18}, C_8[6–8] and other alkylphenyl materials as column packings.

However, the retention data are insufficient to identify components of interest, because of the lack of molecular information. Only newer techniques such as HPLC coupled on-line with ultraviolet–visible spectroscopy (HPLC–UV–VIS) or coupled with mass spectrometry (HPLC–MS) provide good methods for the identification of individual standard compounds in plant extracts[9,10].

The aim of this study was to characterize some extracts of medical plants that are commonly used in cosmetics, using on-line techniques such as HPLC–UV–VIS or HPLC–fluorescence.

EXPERIMENTAL

Equipment

A Hewlett-Packard HP 1090 liquid chromatograph with a DR 5 solvent de-

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livery system, variable-volume auto-injector, auto-sampler, thermostatically controlled column compartment and a microbore column was used for separation. The compounds were identified using an HP 1040 A HPLC detection system, which, by means of diode-array technology measures absorbance at all wavelengths in the range from 190 to 600 nm simultaneously, and an HP 1046A programmable fluorescence detector.

Clean-up was performed using a solid-phase extraction (SPE) system (J. T. Baker, Phillipsburg, NJ, U.S.A.).

Sample preparation

About 150 mg of the individual plant extract were dissolved in 5 ml of distilled water and the solution obtained was applied to a 3-ml Bakerbond octadecyl (C_{18}) SPE cartridge. In order to remove interfering substances such as sugars, sugar colouring and organic acids, the sample was washed with 30 ml of distilled water. The phenolic acids, flavonoids, tannins, alkaloids, etc., were eluted with a mixture of 10 ml of methanol and 0.2 ml of 25% ammonia solution. The eluate was evaporated to dryness, the residue redissolved in 1 ml of methanol and 2–10 μl of the solution were injected.

The clean-up of samples used for the amino acid separation (marshmallow extracts) was performed by dissolving 100 mg of the extract in 2 ml of water and applying the solution obtained to a 3-ml Bakerbond aromatic sulphonic acid cartridge. The adsorbed part of the sample was washed with 2.5 ml of water and eluted with 1 ml of 0.1 *M* hydrochloric acid. The eluate was taken directly for the HPLC separation.

Liquid chromatographic conditions

Flavonoids, phenolic acids, and tannins. The separations of flavonoids, phenolic acid and tannins were performed by gradient elution. Solvent A was phosphoric acid adjusted to pH 2.8 with distilled water and solvent B was acetonitrile. The gradient was controlled by the following time programme:

0–9.9 min: A = 97.5%
10–14.9 min: A = 80.0%
15–16.9 min: A = 65.0%
17–20.0 min: A = 0%

The mobile phase was sparged with helium prior to and throughout the analysis to prevent bubble formation. The flow-rate was 0.5 ml/min. The Hewlett-Packard Hypersil ODS 5-μm microbore column (100 × 2.1 mm I.D.) was maintained thermostatically at 40°C. In flavonoid and phenolic acid analysis, the detector wavelength was set at 337 nm, whereas for *Hamamelis* extracts 274 nm gave best results.

Amino acids. After pre-column derivatization with *o*-phthalaldehyde in the presence of mercaptoethanol, the amino acids were also separated by a gradient elution programme using a Hypersil ODS 5 μm column and the following solvents: A = 0.03 *M* sodium acetate–0.25% tetrahydrofuran (THF)–0.1 m*M* sodium azide in water (pH 7.5); B = acetonitrile–0.1 *M* sodium acetate (80:20)–0.1 m*M* sodium azide. The programme was as follows:

0–9.9 min: A = 98.0%
10–16.9 min: A = 80.0%

17–17.6 min: A = 77.0%
18–25.9 min: A = 70.0%
26–26.9 min: A = 60.0%
27–30 min: A = 0%

The samples were injected and derivatized by an auto-sampler equipped with a microprocessor-controlled unit that carried out successive pipetting, mixing and injection according to a given computer program. The identity of the separated compounds was confirmed by comparison with a chromatogram of the corresponding standard OPA derivatives.

Fluorescence was monitored at an excitation wavelength of 340 nm and at an emission wavelength of 455 nm with a 5-μl flow cell and a xenon lamp.

Cinchonia alkaloids. The separation of the four alkaloids was performed using the binary solvent system 6.3 m*M* aqueous dipotassium hydrogenphosphate solution–methanol (45:55, v/v) under isocratic conditions. The substances were detected most sensitively at 230 nm.

Chemicals

Acetonitrile and water used for the mobile phase were of HPLC grade.

Standard samples were purchased from C. Roth (Karlsruhe, F.R.G.) and from E. Merck (Darmstadt, F.R.G.).

Aqueous extracts of the different plant species were taken from typical industrial production batches.

RESULTS AND DISCUSSION

Table I lists the retention times and absorption maxima of all compounds recognized in the plant extracts in this study. The simultaneous analysis of these compounds with marked differences in polarity requires the use of a gradient elution programme, except for the separation of the *Cinchona* alkaloids. The identity of the registered peaks was confirmed first by comparing the observed retention times of the peaks with those of the individual standard solutions. Further peak characterization was effected by means of a diode-array detector. The UV spectra of the compounds listed in Table I coincide well with those of the pure compounds.

The constituents found in the described plant materials are chemically well defined. However, in *Hamamelis virginiana* the nature of the gallotannins has not yet been completely investigated[11]. In the extract of *Hamamelis* leaves, three different compounds which show very similar UV spectra could be detected (UV absorption at 274 nm): gallic acid, hamamelitannin and one other uncharacterized tannin compound (Fig. 1). The simultaneous determination of these compounds offers the possibility of monitoring the hydrolysis of the hamamelitannin and the formation of gallic acid during the industrial production process and during the storage of the drug, respectively.

Aqueous extracts of *Matricaria chamomilla* flowers have been investigated by HPLC, several different mobile phases being evaluated[12–17]. Some biologically active and/or characteristic constituents such as chlorogenic acid, caffeic acid, umbelliferone, luteolin-7-O-glucoside, apigenin-7-O-glucoside, herniarin and apigenin have been found. However, in our opinion no useful method has yet been found for achiev-

TABLE I

ABSORPTION MAXIMA AND RETENTION TIMES OF THE DETECTED COMPOUNDS

Peak No.	*Compound*	*Absorption maxima (nm)*	*Retention time (min)*
1	Gallic acid	271	1.2
2	Hamamelitannin	274	4.7
3	Tannin derivative (not specified)	270	6.0
4	Chlorogenic acid	216, 322	4.5
5	Caffeic acid	216, 320	4.6
6	Umbelliferone	320	6.4
7	Luteolin-7-O-glucoside	252, 262, 344	9.1
8	3,4-Dicaffeoylquinic acid	323	9.6
9	Apigenin-7-O-glucoside	266, 334	10.2
10	4,5-Dicaffeoylquinic acid	225	10.8
11	Herniarin	320	11.1
12	Apigenin	264, 334	13.9
13	5-Caffeoylquinic acid	217, 323	3.0
14	4-Caffeoylquinic acid	283, 325	4.8
15	Vitexin (Luteolin-8-C-glucoside)	267, 335	8.4
16	Luteolin	250, 262, 346	12.6
17	Labiatic acid	326	10.4
OPA derivatives of:			
18	Asparaginic acid	–	1.6
19	Asparagine	–	5.6
20	Alanine	–	9.8
21	Cinchonidine	225, 285	3.4
22	Cinchonine	225, 285	3.7
23	Quinine	230, 326	4.8
24	Quinidine	230, 326	5.3

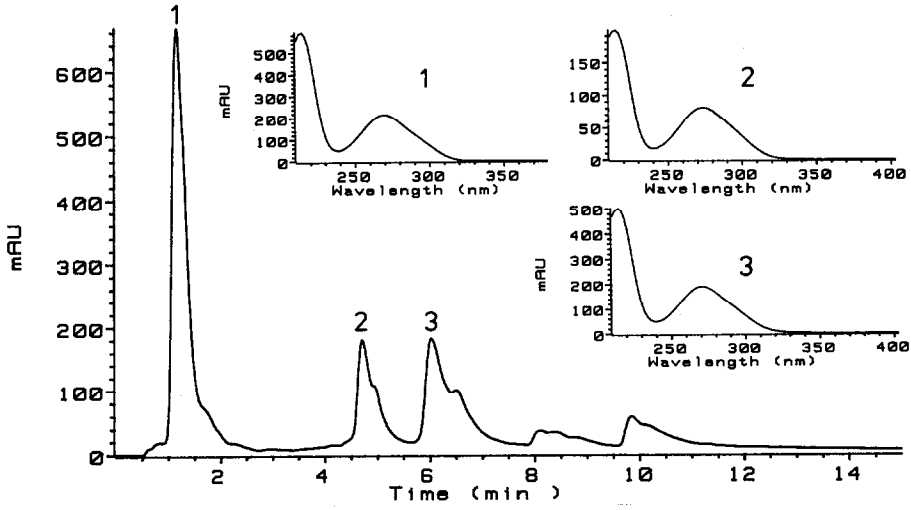

Fig. 1. Reversed-phase HPLC and UV–VIS spectra of an aqueous extract of *Hamamelis virginiana*. Assignment of peak numbers according to Table I. Mobile phase: phosphoric acid (pH 2.8)–acetonitrile, gradient elution programme.

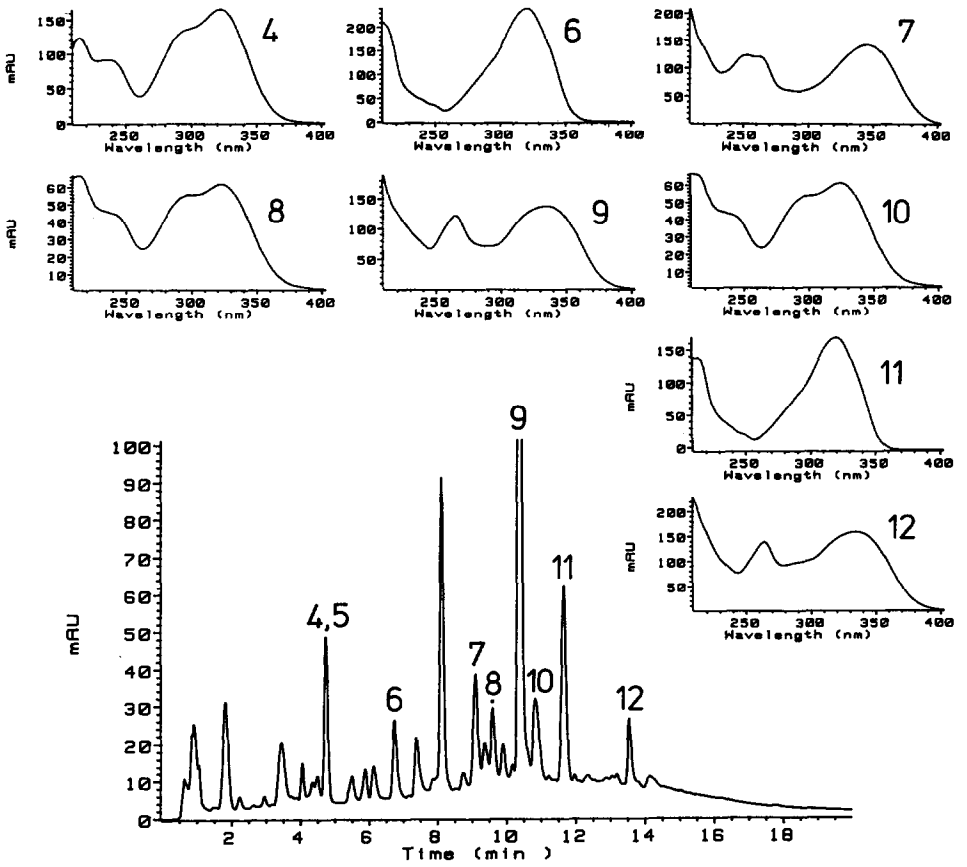

Fig. 2. Reversed-phase HPLC and UV–VIS spectra of an aqueous extract of *Matricaria chamomilla*. Assignment of peak numbers according to Table I. Mobile phase as in Fig. 1.

ing both a high-resolution separation of the characteristic known ingredients and characterization of the unknown compounds by diode-array detection, as illustrated in Fig. 2.

To our knowledge, there are no reports concerning the HPLC determination of substances in aqueous extracts of milfore (*Achillea millefolium*). Usually the drug was extracted with organic solvents such as methanol, light petroleum or acetone. The individual components were separated by TLC or column chromatographic methods and characterized by spectral studies[18–20]. In the aqueous extract of milfore analysed in this study, chlorogenic acid and its isomers (4- and 5-caffeoylquinic acid), dicaffeoylquinic acids, luteolin, luteolin-7-O-glucoside, apigenin-7-O-glucoside and vitexin were detected and characterized both by retention time and by comparison of the UV–VIS spectra with those of the individual standard compounds (Fig. 3). The assignment of the caffeoyl- and dicaffeoylquinic acids was performed according to the well described determination of chlorogenic acids in instant coffee[21]. Moreover, by heating 3-caffeoylquinic acid in dilute ammonia solution at pH 8 for 1 h an equilibrium mixture of the 3-, 4- and 5-isomers could be produced for reference studies.

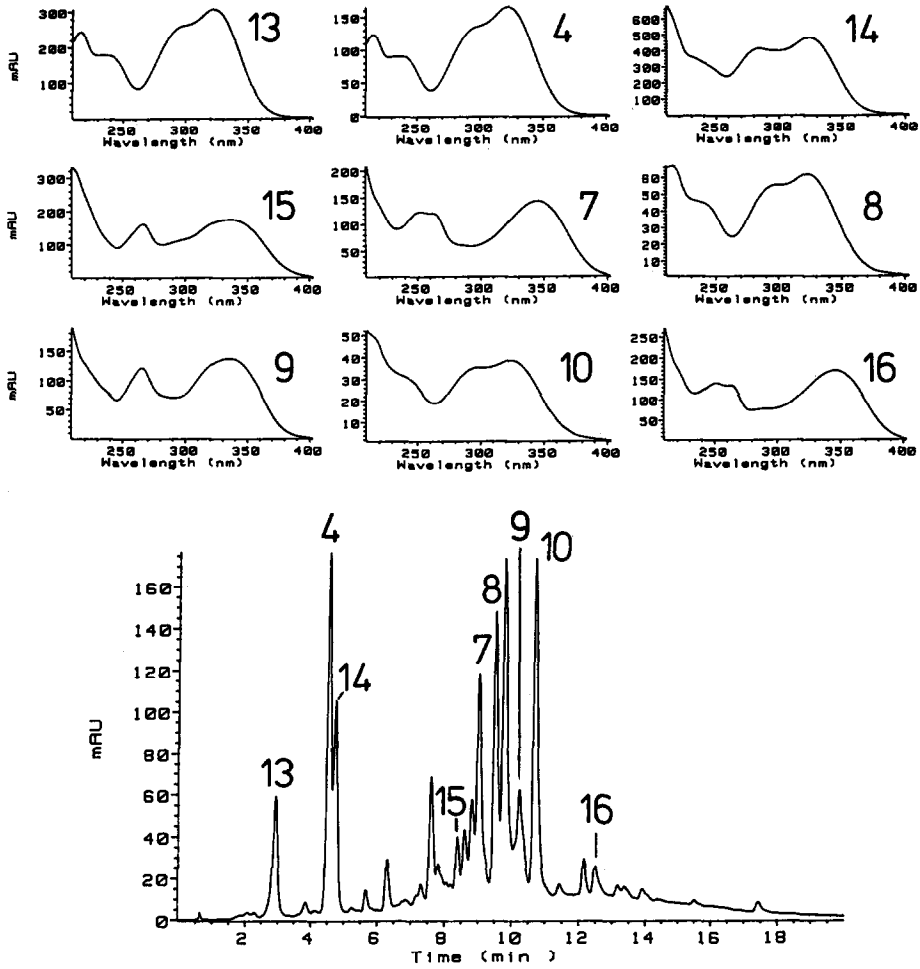

Fig. 3. Reversed-phase HPLC and UV–VIS spectra of an aqueous extract of *Achillea millefolium*. Assignment of peak numbers according to Table I. Mobile phase as in Fig. 1.

Whereas the composition of the essential oils of *Mentha* spp., lavandula (*Lavandula angustifolia*), thyme (*Thymus vulgaris*), rosemary (*Rosmarinus officinalis*) and sage (*Salvia officinalis*) have been well characterized by high-resolution gas chromatography–mass spectroscopy (HRGC–MS) methods, little information is available concerning the hydrophilic components occurring in these plants[22–25].

In all of the above-mentioned species, which belong to the *Labiatae* family, labiatic acid was identified by HPLC–UV–VIS as the main component. Moreover, in *Thymus vulgaris*, in addition to chlorogenic acid, three flavonoids (luteolin, luteolin-7-O-glucoside and apigenin-7-O-glucoside) could be detected (Fig. 4). The chromatographic pattern of the other mentioned aqueous *Labiatae* extracts look very similar, so that it was decided not to present these separations in detail here.

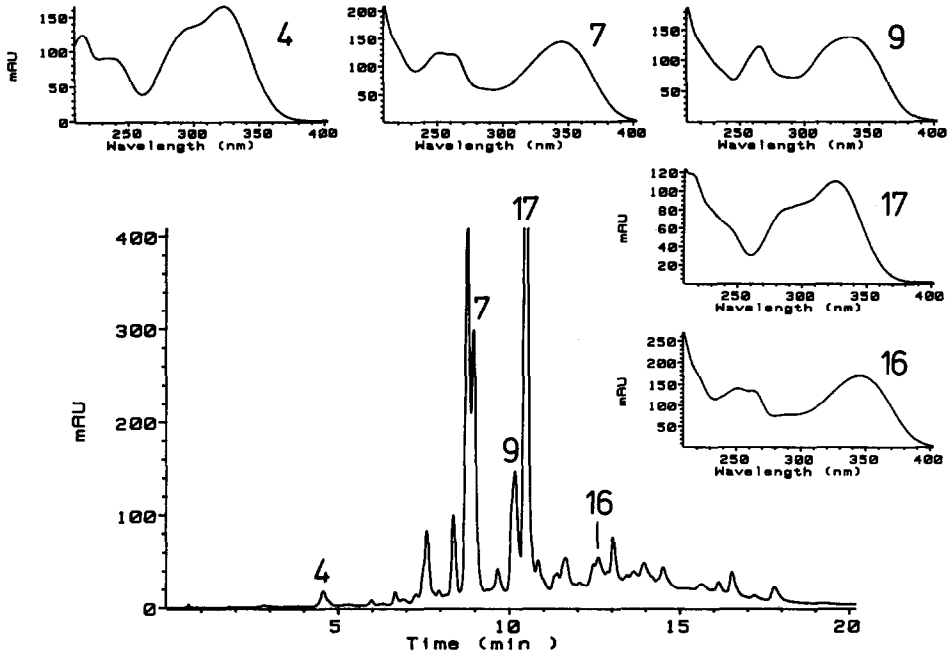

Fig. 4. Reversed-phase HPLC and UV–VIS spectra of an aqueous extract of *Thymus vulgaris*. Assignment of peak numbers according to Table I. Mobile phase as in Fig. 1.

The main water-soluble part of the roots, leaves and flowers of marshmallow (*Althaea officinalis*) consists of mucilages, which had previously been investigated qualitatively and quantitatively[26]. The analytical detection of these polysaccharides is very time consuming and indirect[27], and therefore attempts should be made to

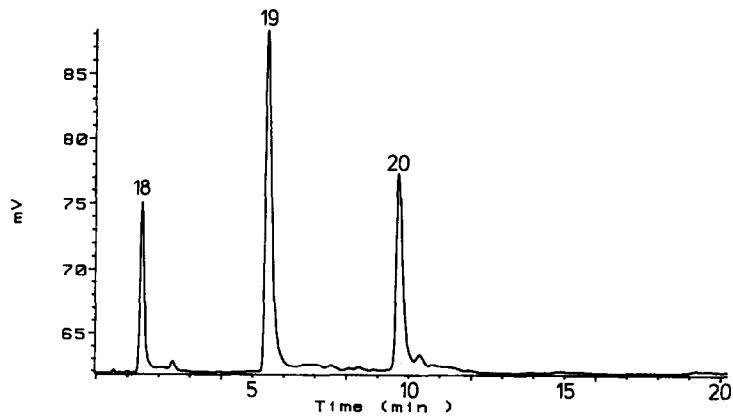

Fig. 5. Reversed-phase HPLC of the amino acids detected in an aqueous extract of *Althaea officinalis* using *o*-phthalaldehyde pre-column derivatization. Assignment of peak numbers according to Table I. Mobile phase: sodium acetate–THF–acetonitrile, gradient elution programme.

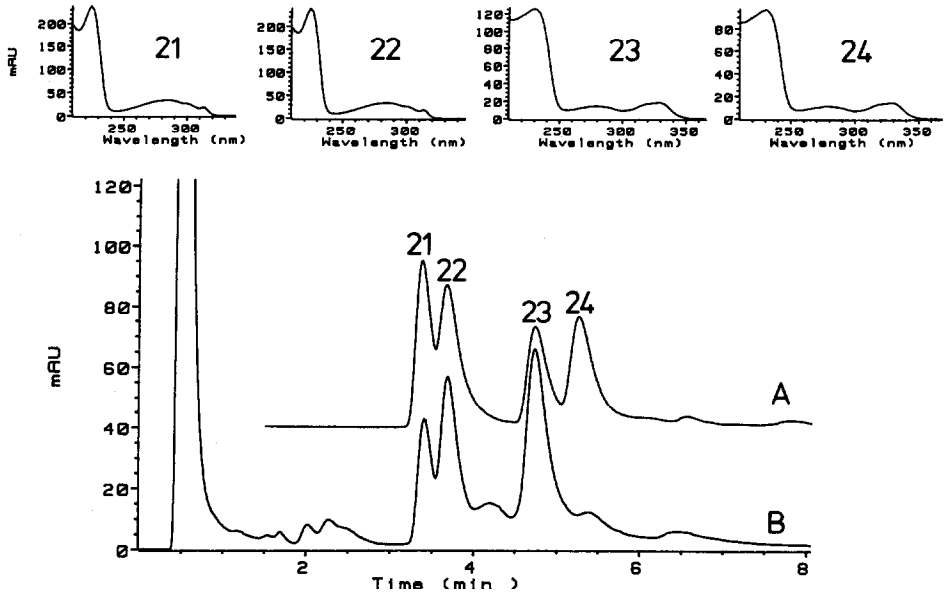

Fig. 6. Reversed-phase HPLC and UV–VIS spectra of a mixture of alkaloid standards (A) and an aqueous extract of *Cinchonia* spp. (B). Assignment of peak numbers according to Table I. Mobile phase: 6.3 m*M* K_2HPO_4–methanol (45:55, v/v), isocratic.

find other characteristic ingredients. Nowadays the analysis of amino acids can be performed very rapidly and with high sensitivity by automatic pre-column derivatization methods[28–30]. Using this procedure, the composition and individual contents of free amino acids in plant materials can be examined very successfully. Fig. 5 shows the chromatographic separation of the OPA derivatives of alanine, asparagine and asparaginic acid, detected in an aqueous extract of *Althaea officinalis*.

The analysis of the four major alkaloids from the bark of *Cinchona* spp. (quinine, quinidine, cinchonine and cinchonidine) has received considerable attention because of the wide pharmaceutical use of this drug. Several HPLC methods have been developed for the separation of these compounds but most of them give insufficient separation, need long separation times or use a ternary or a quaternary solvent system[31–34]. Using a microbore reversed-phase column in combination with a binary solvent system of dipotassium hydrogenphosphate solution and methanol, complete resolution of the four alkaloids can be achieved within a few minutes, as shown in Fig. 6.

In conclusion, the methods described are useful approaches for characterizing a number of medical plants by their HPLC "fingerprints" and by quantification of their characteristic ingredients.

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