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Qualitative and quantitative micellar electrokinetic chromatography of kavalactones from dry extracts of *Piper methysticum* Forst. and commercial drugs☆

Matthias Lechtenberg[a], Bettina Quandt[a], Franz-Josef Kohlenberg[b], Adolf Nahrstedt[a,*]

[a]*Institut für Pharmazeutische Biologie und Phytochemie, Hittorfstraße 56, D-48149 Münster, Germany*

[b] *Institut für Pharmakologie und Toxikologie, Domagkstraße 12, D-48149 Münster, Germany*

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Abstract

A rapid micellar electrokinetic chromatography method with diode-array detection has been developed for the identification and quantitative determination of the therapeutically important styrylpyrones from *Piper methysticum* extracts and commercial drugs. Using fused-silica capillaries with a borate buffer containing sodium-taurodeoxycholic acid and β-cyclodextrin kavain, dihydrokavain, methysticin, dihydromethysticin, yangonin and demethoxyyangonin were fully separated and quantified within 15 min with 4-hydroxybenzoic acid methyl ester as I.S.

Keywords: Piper methysticum; Pharmaceutical analysis; Plant materials; Kavalactones; Styrylpyrones

1. Introduction

Piper methysticum Forst. (Piperaceae) has been cultivated by the natives of the South Pacific islands for more than 3000 years. For the preparation of the beverage ''kava kava'' the roots and rhizome were chewed or crushed and suspended in water. Traditionally this preparation was drunk at ceremonial events because of its well-known tranquilizing and calming properties [1]. Pharmacological investigations have shown sedative, muscle relaxant, analgesic [2], anticonvulsive, anesthetic [2,3], antiarrhythmic [4], antithrombotic [5], neuroprotective and antispasmodic [3] effects. Several clinical trials have proven an anxiolytic activity in humans [6,7]. Therefore, kava is generally described as a herbal alternative to benzodiazepines for the treatment of anxiety disorders [7].

The lipophilic kavapyrones are regarded as the pharmacological active constituents of kava. So far 18 kavapyrones have been isolated [8], six of which constitute the major and pharmacologically important components (Fig. 1); they are structurally divided into enolides (chiral α-pyrones **1**–**4**) and dienolides (achiral 5,6-dihydro-α-pyrones **5,6**) [6]. Several HPLC methods for separation and quantification of kava lactones were published. Silica gel [9–11], aluminia [11] and RP phases [11–13] were used to separate crude drug extracts and standard mixtures of

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*Corresponding author.

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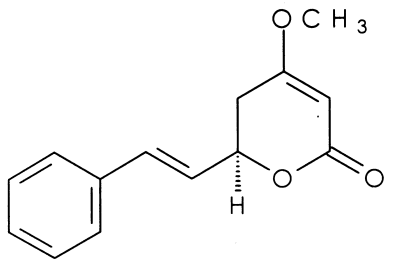
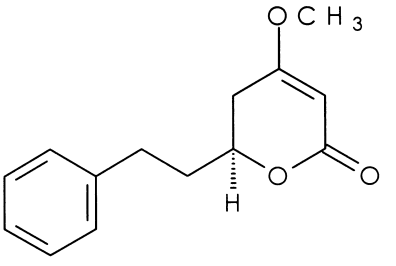
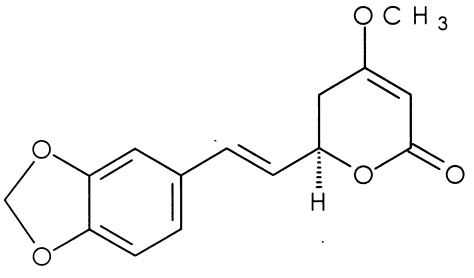


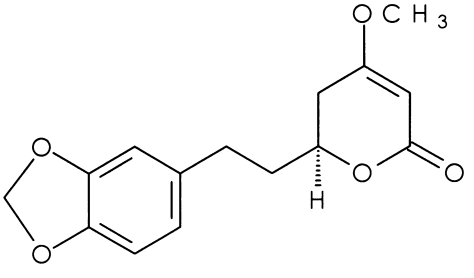
1 (+)-Kavain (K)



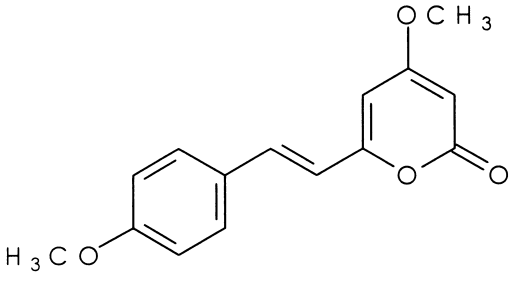
2 (+)-Dihydrokavain (DHK)



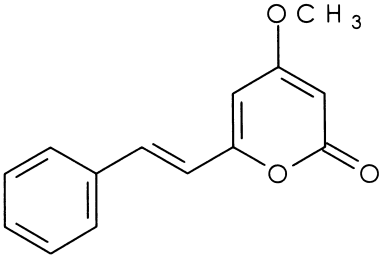
3 (+)-Methysticin (M)



4 (+)-Dihydromethysticin (DHM)



5 Yangonin (Y)



6 Demethoxyyangonin (DMY)

Fig. 1. Six major styrylpyrones from the roots and rhizome of *Piper methysticum*.

the major kavalactones (+)-kavain **1**, (+)-dihydrokavain **2**, (+)-methysticin **3**, (+)-dihydromethysticin **4**, yangonin **5** and demethoxyyangonin **6**. An enantiomeric separation on a poly-(*S*-methyl-*N*-acryloylphenylalanine) column was reported [14,15]. Separation of **1–6** was obtained within 17–40 min with normal and reversed-phases and up to 100 min for chiral separation [8–15].

However, short time separation using normal and reversed-phases was comparatively poor [11] and good separations usually required more than 30 min time [9,12–15]. Thus, we developed a new method using capillary electrophoresis (CE) that, in comparison to HPLC, is simpler, faster, more convenient and has a higher resolving power for complex multicomponent mixtures (e.g. [16]).

2. Experimental

2.1. Plant material and commercial drugs

Three different dry extracts of *Piperis methystici* rhizoma were obtained from Krewel Meuselbach, Eitorf, Germany: K1 (15.3% kavapyrones), K2 (30.6% kavapyrones), K3 (HPLC-validated standard with 27.46% kavapyrones). Commercial drugs were obtained from German pharmacies.

2.2. Reagents and standards

H_3BO_3 (article 1.06306) and $Na_2B_4O_7$ (article 1.00165) were obtained from E. Merck, Darmstadt, Germany; sodium taurodeoxycholate monohydrate (article 1180-95-6) was purchased from Fluka, (Buchs, Switzerland); β-cyclodextrin was a gift of Professor G. Blaschke (University of Münster, Germany); methanol was of HPLC quality, water was prepared by two-step distillation of demin. water. Before use all liquids were filtered through nylon filters (0.22 μm, article E256.1, Carl Roth, Karlsruhe, Germany). The first 1–2 ml of the eluate were discarded.

4-Hydroxybenzoic acid methyl ester as I.S. (article 4748, Rotichrome) was obtained from Carl Roth; the I.S. solution contained 10.0 mg/ml. D/L-Kavain was a gift from Krewel Meuselbach. The enantiomeric pure compounds **1**–**6** were purchased from Addipharma, Hamburg, Germany.

2.3. Apparatus

Analyses were performed on a P/ACE 5010 instrument (Beckman Instruments, Palo Alto, CA, USA) with photodiode-array detection. Electropherograms were recorded and integrated using an interfaced personal computer with the software ''System Gold'' (Beckman Instruments).

2.4. Separation parameters

An uncoated fused-silica capillary [77 cm (70 cm to detector)×50 μm I.D.] was used. Before analysis, the capillary was equilibrated with running buffer (2 min). After analysis, the capillary was washed with 0.1 *M* NaOH (2 min). New capillaries were washed with running buffer for several hours until reproducible migration times were obtained.

The running buffer was composed of 50 m*M* sodium taurodeoxycholate in 100 m*M* borate buffer (100 m*M* H_3BO_3: 100 m*M* $Na_2B_4O_7$ 9:1; pH 8.3) containing 0.75% (m/V) β-cyclodextrin. The pH value of the complete running buffer was 7.9. The injection time was 1–2 s at 0.5 p.s.i. (1 p.s.i.= 6894.76 Pa). Detection was at 240 nm (for quantification) and 350 nm, voltage was at 30 kV (resulting current=30–31 μA), temperature at 27°C.

2.5. Sample preparation

50–300 mg of dried plant extracts, total contents of capsules, aliquots of ground pills and coated tablets and aliquots of tinctures were dissolved in 20 ml MeOH. 1.0 ml internal standard solution was added, the mixture was then sonicated for 15 min and filled up to 25.0 ml. 200 μl of the filtered solution were used for micellar electrokinetic chromatographic (MEKC) analysis. Samples were stored at 20°C in darkness and analyzed not later than 1 h after preparation.

2.6. Solutions for calibration procedures

Calibration was restricted to four concentration levels (ca. 0.1–0.9 mg/ml) because of lack of sufficient amounts of pure compounds **1–6**, with 0.4 mg/ml I.S. at each level. Compounds **1–6** (1.5–2.5 mg) were weighted on a micro-balance (type 4401, Sartorius, Göttingen, Germany). The calibration curves were obtained by plotting concentrations versus the corrected peak area ratios with *cPA*= corrected peak area, *c*=concentration [mg/ml], I.S.=internal standard [mg/ml]:

$$\frac{cPA_{1-6}}{cPA_{\text{I.S.}}} \cdot c_{\text{I.S.}} = a \cdot c_{1-6} + b \quad (1)$$

Linearity of the kavain calibration was additionally proved with pure D/L-kavain and 4-hydroxybenzoic acid methyl ester as I.S. The concentration of kavain ranged from 0.05–1.6 mg/ml in six steps (0.05/0.1/0.2/0.4/0.8/1.6) with each level containing 0.4 mg/ml I.S.

2.7. Recovery studies (kavain)

Four solutions of 100.0 mg dry kava extract in MeOH were spiked with 0, 10, 20 and 30 mg D/L-kavain and 1.0 ml I.S. solution and filled up to 25.0 ml with each solution containing 0, 0.4, 0.8 and 1.2 mg/ml D/L-kavain, 0.4 mg/ml I.S. and 4.0 mg/ml dry kava extract. The concentration of D/L-kavain in the dry extract was obtained from the cut of the *x*-axis of the linear curve of a plot of the added D/L-kavain vs. the ratios of corrected peak areas of kavain and the I.S.

As no blank matrix of kava kava was available increasing amounts of D/L-kavain were added to a sample solution. After analysis of the kavain amount in the sample solution equal values in comparison with a calculation based on a calibrating factor were achieved.

2.8. Linearity and repeatability

(**1**) The calibration curve (performed with D/L-kavain) showed linearity up to 1.6 mg/ml. The regression index r was at 0.9998. Calibration with enantiomeric pure kavain yielded a calibration factor (slope a in Eq. (1)) of 2.8409 ($r=0.9997$). (**2**): $r=0.9995$, $a=1.4538$; linearity proved up to 0.95 mg/ml. (**3**): $r=0.9996$, $a=1.7770$; linearity proved up to 0.78 mg/ml. (**4**): $r=0.9981$, $a=1.4715$; linearity proved up to 0.71 mg/ml. (**5**): $r=0.9990$, $a=1.0262$; linearity proved up to 0.74 mg/ml. (**6**): $r=0.9999$, $a=1.2681$; linearity proved up to 0.89 mg/ml.

The relative standard deviations (RSD) for intra-day precision (repeatability) are as follows: **1** 1.7%, **2** 1.9%, **3** 2.0%, **4** 2.0%, **5** 2.9%, **6** 2.2%. The RSD values for inter-day precision are: **1** 2.5%, **2** 3.3%, **3** 3.5%, **4** 4.3%, **5** 6.3%, **6** 2.5%

2.9. Limit of quantification (LOQ), limit of detection (LOD)

The LOQ for kavain **1** is 0.05 mg/ml as this is the lowest level of the calibration curve described in 2.6. For the same reason no quantitative predictions at concentration levels below 0.1 mg/ml can be made concerning **2**–**6**. As a practicable way for determination of the LOD [17] we chose the triple baseline noise: 0.01 mg/ml for **1**–**6**.

3. Results and discussion

1–**6** are uncharged molecules under neutral, slightly alkaline and acid conditions indicating that simple capillary zone electrophoresis (CZE) could not be an appropriate method for analysis. Strong basic pH should be avoided because the lactones are not stable under such conditions. Thus, a selective MEKC method was developed which combines advantages of reversed-phase chromatography and capillary electrophoresis. Since the method should allow a rapid baseline separation of all six major kavapyrones in crude extracts, method development was done with a methanolic solution of the dry extract K1 (cf. Section 2.1.).

In preliminary experiments anionic surfactant additives such as sodium dodecylsulfate (SDS), sodium cholate, sodium deoxycholate and sodium taurodeoxycholate (TDCh) in phosphate and borate buffer systems were tested. The concentrations of the micelle-forming agents ranged from 10 to 100 m*M*. Only TDCh in borate buffer solution showed a promising separation. Best results were obtained at buffer concentrations about 100 m*M* and pH-values between 8.3 and 9.2 (pH measured before surfactant was added). For stability reasons the lowest possible pH value was chosen.

In order to optimize the surfactant concentration five buffer systems with increasing TDCh concentrations (10, 25, 50, 75, 100 m*M*) were tested. Increasing TDCh caused an improved separation (Fig. 2) but also a prolonged time of analysis, as k is linearly related to the concentration of the pseudo-stationary phase [18]. However, separation of compounds **1** and **4** could not be achieved even at concentrations of TDCh above 50 m*M*. Therefore,

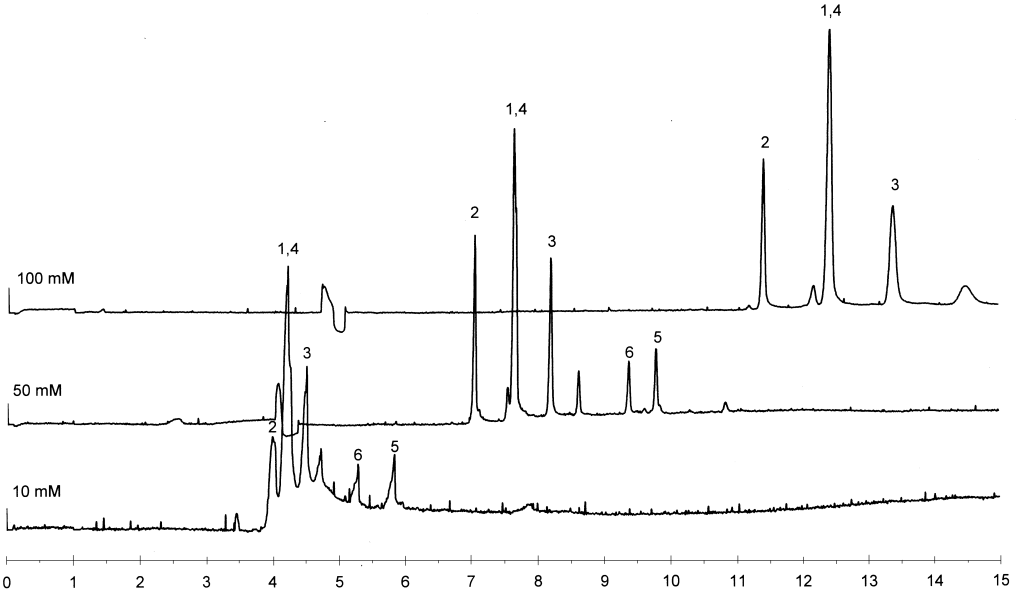


Fig. 2. Influence of TDCh concentration [100 m*M* borate buffer, pH 8.3, 30 kV, capillary: 77 cm (70 cm to detector)×50 μm I.D., 240 nm]. For assignment of the peak numbers see Fig. 1.

surfactant additives and several combinations of anionic micelle-forming agents with and without urea and organic modifier were tested, but without success in separating the co-migrating compounds **1** and **4**.

To enhance selectivity, cyclodextrins (CD) as competing separation principle were added [19,20]. α-, β- and γ-CD as well as hydroxypropyl-β-CD, ethyl-β-CD, mono-, di- and trimethyl-β-CD were investigated, but only β-CD improved the separation at concentrations between 0.25 and 4% β-CD (tested: 0.25, 0.5, 0.75, 1, 2, 3, and 4%). Parts of the results are shown in Fig. 3. Already at 0.25% (m/v) β-CD all six major kava lactones (**1**–**6**) could be well separated. Above 0.5% β-CD compounds **4** and **3** could be separated from the hitherto unidentified compounds **A** and **D** which showed absorption at 240 nm. When increasing the concentration of β-CD the separation factor α of compounds **2** and **4** was shifted to unsatisfying values (1.10 to 1.03 from 0 to 1% of β-CD); consequently, the conditions as given in 2.4 with 0.75% β-CD were used for separation.

Fig. 4 shows the MEKC-analysis of a methanolic extraction of the crude extract K1. Compounds **1**–**4** and **A**–**D** exhibit UV-absorption at 240 nm and almost no absorption at 350 nm, compounds **5**, **6** and **E**–**H** absorbed at both wavelengths with a higher intensity at 350 nm. Table 1 shows the retention factor *k* for each kavapyrone and the prominent undetermined compounds. The signals of compounds **1**–**6** were tested for peak purity by their UV spectra between 200 and 500 nm. No difference was visible in comparison to the standard compounds **1**–**6** indicating that the peaks were pure.

Different extraction time (5, 15, 30 and 60 min) of the crude drug material was tested in an ultrasonic bath to verify a complete extraction. Above 15 min no further improvement was observed. When methanolic solutions of K1 were stored in a water bath at 40°C for 24 h a slight decline of **2**–**6** (<5%) and a loss of 12% of **1** were observed in comparison to a methanolic solution stored at 20°C. When methanolic solutions of K1 were exposed to white light (9500 Lux) for 24 h compounds **1**–**4** and **6** only slightly decreased, whereas yangonin **5** showed loss of 56% in comparison to a reference solution stored in the dark. Concomitantly, the hitherto undetermined compound **E** increased to a level of almost 1:1 with compound **5**, indicating that **5** and **E** probably are E/Z-isomers. Such behaviour was already observed by Smith et al. [11] who reported that trans-yangonin is converted to a mixture of cis/trans-yangonin after

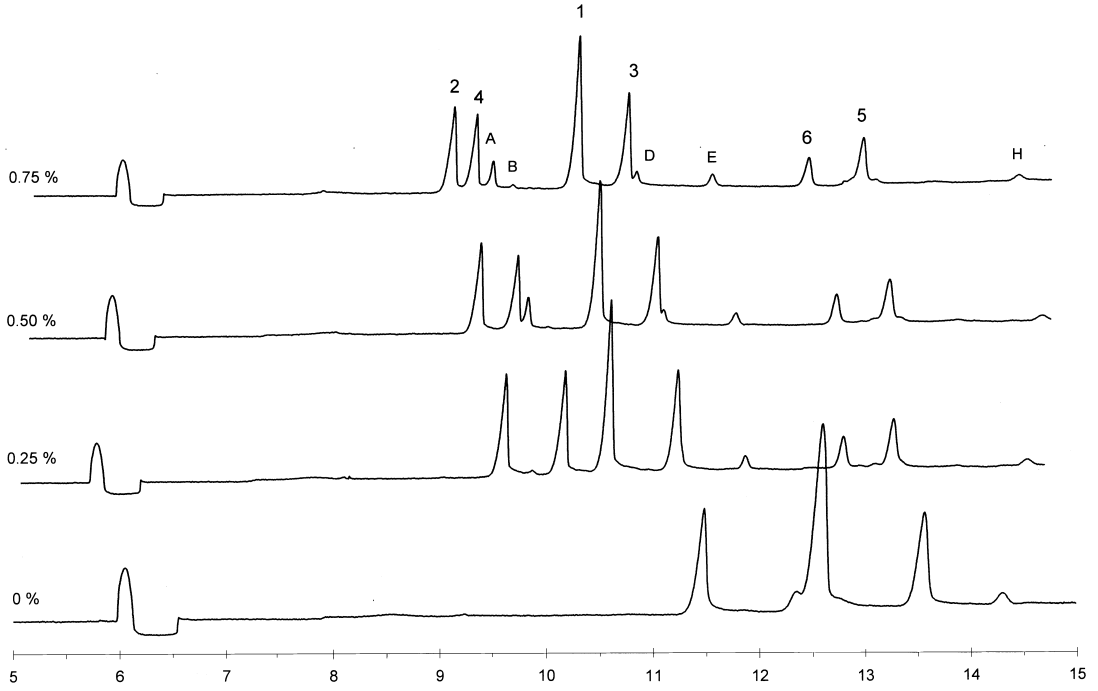


Fig. 3. Influence of β-CD concentration [100 m*M* borate buffer, pH 8.3, 50 m*M* TDCh, 30 kV, capillary: 77 cm (70 cm to detector)×50 μm I.D., 240 nm]. For assignment of the peaks see Fig. 1 and text.

exposure to daylight in organic solvents. In 1997 He et al. [8] also observed a ''yangonin isomer'' that increased in time during HPLC/MS of crude extracts. Thus, samples were stored at 20°C protected from light and used for analysis not longer than 1 h after preparation.

Although the commercial drugs and dry extracts analyzed here contained inactive ingredients such as Ca_2HPO_4, lactose, saccharose, VP, PVP, SiO_2, magnesium stearate and propylene glycol, the electropherograms obtained from the samples showed no interferences due to the additives present.

Besides the dry extracts K1, K2 and K3 some commercially available preparations of the crude drug of *Piper methysticum* were analyzed and the sum of **1–6** (in brackets) was compared to the labelled value of the manufacturers. Capsules were declared as 47.5–52.5 mg/capsule kavapyrones (own result was 51.6 mg/capsule); tablets: 120 (122.8) mg/tablet; coated tablets 10.0–10.9 (10.3) mg/coated tablet; tincture: 25 (24.7) mg/ml; validated dry extract K3: 27.47 (27.2) g/100 ml. Altogether, the results with the present method are in good accordance with the declared values most of which were obtained by HPLC methods.

4. Conclusion

The new method provides a selective and rapid determination for analysis of the six major kavapyrones from crude extracts and drug preparations of the rhizome and roots of *Piper methysticum*. The method is robust (cf. Section 2.8), sensitive enough for a standard method and recovery is good (cf. Section 2.7). According to our knowledge this is the first publication of a qualitative and quantitative analysis of kava kava material using capillary electrophoresis as separation technique. In comparison to the usually used silica-gel HPLC the proposed method is slightly faster, but not affected by water in the sample solution providing a quick and comfortable sample preparation, especially when aqueous systems have to be investigated, e.g. during phar-

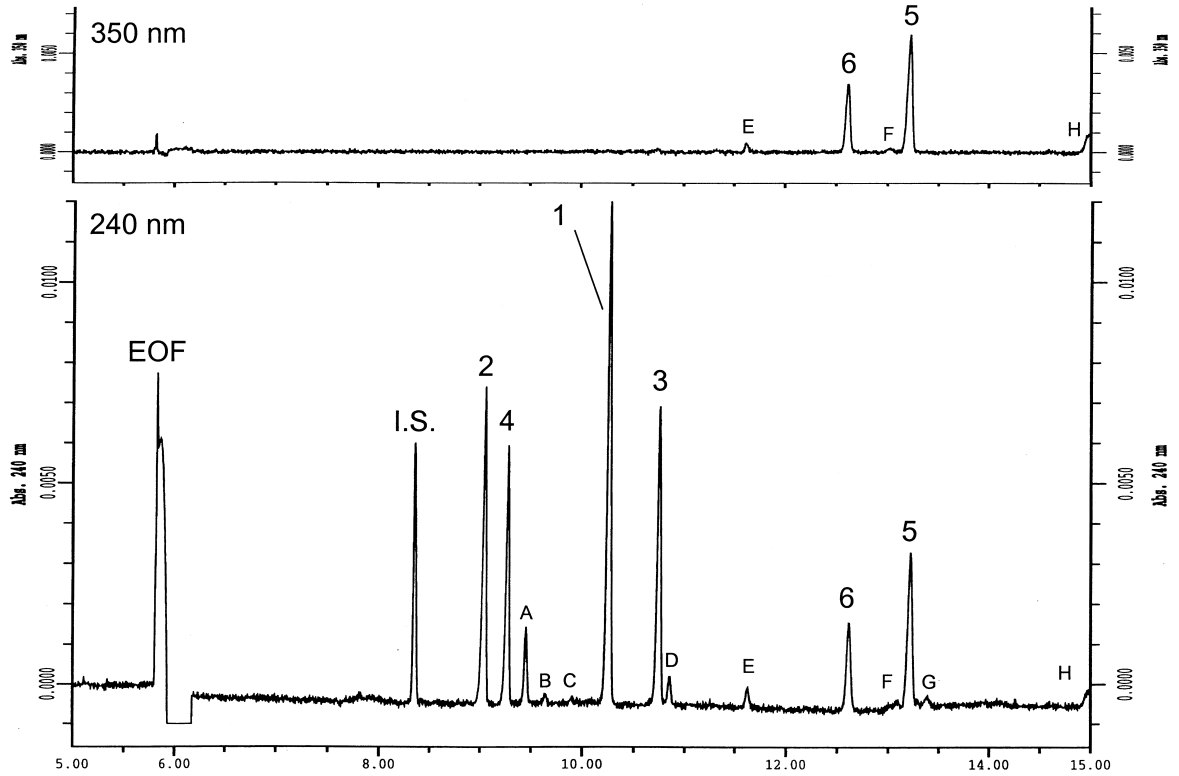


Fig. 4. MEKC separation of *Piper methysticum* dry extract K1 [solution in methanol, 9 mg/ml, 100 m*M* borate buffer, pH 8.3, 50 m*M* TDCh, 0.75% β-CD, 30 kV, capillary: 77 cm (70 cm to detector)×50 μm I.D.]. For assignment of the peaks see Fig. 1 and text.

Table 1
Retention factors *k* of styrylpyrones **1**–**6** and minor unidentified components **A**, **D**, **E** and **H**. Methanol was used to determine the EOF, sudan III to determine migration of the taurodeoxycholate micelles

Kavapyrones	Undet. comp.	Marker	*k*
		EOF	0.00
		I.S.	0.89
2			1.32
4			1.47
	A		1.59
1			2.24
3			2.73
	D		2.85
	E		3.95
6			6.09
5			8.21
	H		39.35
		Sudan III	∞

macokinetic studies. Comparing to RP-HPLC [8,12], a baseline separation of the major pyrones is achieved within 15 min which is less than half the time of equally effective HPLC methods; a better resolution is obtained indicated by eight unidentified minor peaks (Fig. 4, **A**–**H**) which are only partly or even not detected from crude extracts in the HPLC systems [8–15].

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