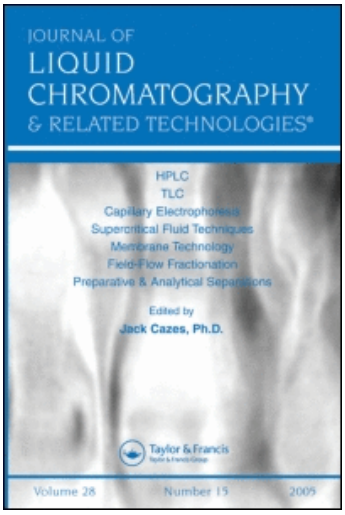

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Application of High Speed Countercurrent Chromatography (HSCCC) to the Isolation of Kavalactones

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Abstract: High speed countercurrent chromatography (HSCCC) was used to isolate the major kavalactones kavain, 7,8-dihydrokavain, methysticin, 7,8-dihydromethysticin, yangonin, and demethoxyyangonin. An ethanolic crude extract of kava root was subjected to HSCCC separation. Due to the presence of polymeric material, the sample load was limited. Hence, gel chromatography was applied in order to concentrate the kavalactones and to reduce the amount of polymers. The purified extract was again subjected to a HSCCC separation. A higher sample input was achieved, but only kavain and demethoxyyangonin were obtained in pure form. The remaining kavalactones eluted in three fractions, which needed to be further purified by preparative HPLC.

Keywords: Kavalactones, Kava kava, High speed countercurrent chromatography (HSCCC), Gel chromatography, Preparative HPLC, 5,6-Dihydroyangonin, Trans-yangonin

INTRODUCTION

Kavalactones are the main bioactive constituents of the kava kava plant (*Piper methysticum*), which is generally cultivated for its rootstock on islands of the South Pacific like Fiji, Tonga, Samoa, and Vanuatu. The indigenous population has been using the root for many centuries to prepare the traditional kava beverage, which is consumed on ceremonial occasions, as well as private gatherings, because of its psychoactive properties. Furthermore, the

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native people have used various parts of the plant for treatment of a number of diseases.[1,2]

Due to the pharmacological properties (sedative, analgesic, muscle-relaxant, and anxiolytic effects), the kava plant was the subject of extensive research during the last century. As a result, the so-called kavalactones were identified as the active compounds, which are classified as substituted α-pyrones or substituted 5,6-dihydro-α-pyrones. The main constituents are 7,8-dihydrokavain (1), kavain (2), demethoxyyangonin (3), 5,6,7,8-tetrahydroyangonin (4), yangonin (5), 7,8-dihydromethysticin (6), and methysticin (7)[1,2] (Figure 1).

Until June, 14th 2002, kava preparations were legally sold in Germany as approved phytopharmaceuticals for the treatment of anxiety and restlessness.[3] More recent reports on the potential hepatotoxic activity of kava extracts, however, prompted the BfArM (Bundesinstitut für Arzneimittel und Medizinprodukte) to withdraw the approval of kava preparations.[4] Other countries followed and restricted the selling of kava preparations as well.[5]

Kavalactones have been extensively investigated with various analytical methods like TLC,[6] HPLC,[7–9] HPLC-MS,[10] GC-MS,[11] and NMR spectroscopy.[12] Due to coelution, the isolation procedure of the kavalactones has always been time consuming and complicated. In the past, repeated column chromatography with silica as stationary phase seemed to be the only useful method to achieve relatively large amounts of pure compounds.[8,12] More recently, Mikell et al., 2003[13] described the isolation of the kavalactones applying high performance centrifugal partition chromatography (HPCPC). The collected fractions were analyzed by TLC on silica. Although this method yielded pure compounds, large quantities of solvents had to be used. Besides HPCPC, no other liquid-liquid chromatographic technique has been described for the isolation of kavalactones so far.

The aim of this work was to find a rapid and easy method for the isolation of the major kavalactones. Moreover, the amounts of solvents used should be minimized and the yields of the isolated pure compounds high enough to enable their use as standards for biological tests. In this paper, we report on the application of high-speed countercurrent chromatography (HSCCC) as a chromatographic technique, which proved to be a useful tool in cleaning up kava kava extracts. HSCCC has already been shown to be an advantageous means for the isolation of other natural products like flavonoids, flavonoid glycosides, phenolic glycosides, coumarins, and more lipophilic compounds like diterpenes, etc.[14]

EXPERIMENTAL

Materials

Dried kava roots were purchased from Alfred Galke GmbH (Gittelde, Germany) in 2002.

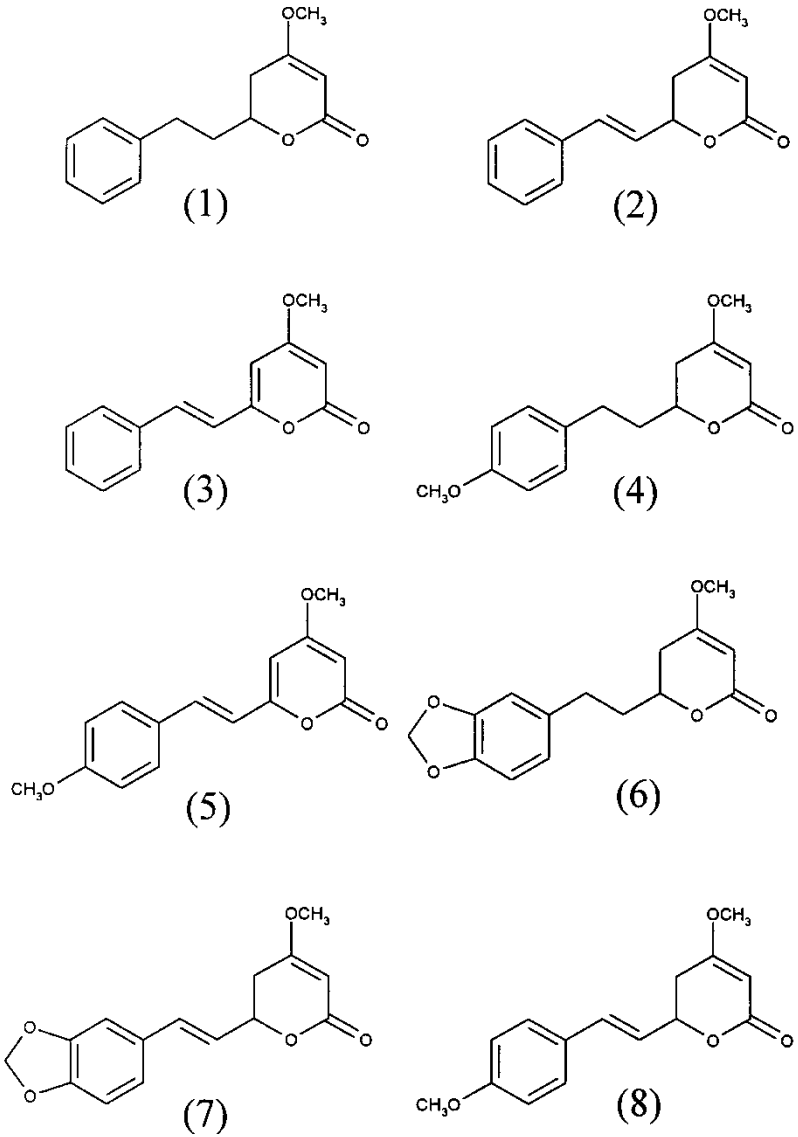

Figure 1. Structures of isolated kavalactones from kava kava. 7,8-Dihydrokavain (1), kavain (2), demethoxyyangonin (3), 5,6,7,8-tetrahydroyangonin (4), trans-yangonin (5), 7,8-dihydromethysticin (6), methysticin (7), 5,6-dihydroyangonin (8).

Solvents of HPLC and p.A. quality were purchased from Fisher Scientific (Loughborough, UK) and Sigma-Aldrich (Steinheim, Germany). Water was purified with a nanopure water purification system (Nanopure, Barnstead). Crude methanol was distilled in our lab before use. Chloroforme-d

was purchased from Armar (Döttingen, Switzerland). The TLC plates (aluminium sheets, silica gel 60 F_{254}) were from Merck (Darmstadt, Germany).

Sample Preparation

The dried kava roots were ground in a mill (Retsch SM 1, Germany). The kava powder was divided into two portions: about 200 g of the powdered root was extracted with acetone at room temperature twice, for four hours. The acetone extracts were filtered, respectively, combined, concentrated in vacuum, and stored in a freezer at $-21^{\circ}C$. The yield was 5 g. Another 200 g of the powdered kava material was extracted with ethanol for one hour at $60^{\circ}C$. After filtration, the procedure was repeated with the filtrate. The combined extract was concentrated in vacuum and stored in a freezer at $-21^{\circ}C$ (5.5 g).

The two obtained crude extracts were analyzed by normal phase HPLC. HPLC-MS analysis enabled the characterization of the different compounds (chromatograms not shown). The ethanolic extract was used for the HSCCC separation.

Gel Chromatography

The acetone extract was subjected to gelchromatography (Sephadex LH-20 column, length: 88 cm, width: 5 cm, Sephadex LH-20 material was conditioned with 100% methanol before use). The extract was dissolved in a small amount of mobile phase (100% methanol) and the sample was put in small fractions on the column. The solvent was always drained before putting the next part of the sample on the column to minimize tailing of the extract. The valve of the column was opened to result in a dropping speed of about one droplet per minute. The obtained thirty-minute fractions were collected with a Pharmacia LKB Super Frac fraction collector and monitored by TLC plate on silica gel. The solvent system was n-hexane/ethyl acetate 1:1 (v/v), the TLC was developed once and sprayed with anisaldehyde/sulfuric acid spraying reagent afterwards, and heated until the coloration of the different spots was apparent. The thirty-minute fractions were combined according to TLC results. Fraction III yielded 4.5 g.

HPLC

The analytical normal phase HPLC experiments were carried out using a binary Knauer HPLC Pump 64 with a Knauer Variable Wavelength Monitor, set to various wavelengths (246 nm-288 nm), driven by Knauer

HPLC Software. The column was a Si Eurospher 100, Merck (Berlin, Germany), with a 250 mm × 4.00 mm I.D. and 5 μm particle size. The mobile phase was an isocratic system consisting of n-hexane/p-dioxane (85:15, v/v). The flow rate was 1.5 mL/min.

For the preparative normal phase HPLC experiments, a binary Knauer HPLC Pump 64 with a Knauer Variable Wavelength Monitor and a LKB, Bromma 2210, Two Channel Recorder were used. The separations were carried out using a Si Eurospher 100 column (5.0 μm particle size, 250 mm × 16 mm I.D.) with a precolumn (LiChrospher, Si 60) from Merck (Berlin, Germany). Demethoxyyangonin was purified with the following isocratic system: n-hexane/ethyl acetate (7:2, v/v) with a flow rate of 6 mL/min; for 7,8-dihydrokavain and trans-yangonin, 7,8-dihydromethysticin and 5,6,7,8-tetrahydroyangonin, methysticin and 5,6-dihydroyangonin n-hexane/p-dioxane (85:15, v/v) with a flow rate of 8 mL/min was used, respectively.

High Speed Countercurrent Chromatography (HSCCC)

A high speed model CCC-1000 manufactured by Pharma Tech Research Corp. (Baltimore, MD, USA) was used. It consisted of three serial preparative coils with a total coil volume of 800 mL. The solvent system n-hexane/ethyl acetate/methanol/water (6:5:6:5, v/v/v/v) was used in the head-to-tail modus with the upper phase as stationary phase. The revolution speed was set to 1000 rpm. The solvents were pumped into the system with a Biotronik HPLC pump BT 3020 at a flow rate of 3.0 mL/min. Varying amounts of sample, 450 mg to 1000 mg of the ethanol crude extract (HSCCC-1), and 820 mg of the cleaned up acetone extract (HSCCC-2) were used, respectively. The samples were dissolved in equal amounts of upper and lower phase (totaling 25 mL) and injected with a loop. The separation was monitored with a Knauer UV K-2501 detector, which was set to 246 nm and the chromatogram was recorded with a Servogor 120, BBC Goerz Metrawatt plotter. Twelve-milliliter fractions were collected with a Pharmacia LKB Super Frac fraction collector.

After the separation was completed, the content of the coil was emptied and the volume measured. The retention of stationary phase was calculated by dividing the volume of the stationary phase (upper phase) with the total coil volume and multiplying by 100.

The partition coefficients were calculated by subtracting the volume of the displaced stationary phase from the eluted sample volume and dividing the difference between total coil volume and displaced stationary phase.

$$k = \frac{V(RT) - V(SF)}{V(TOT) - V(SF)}$$

k = partition coefficient

V(RT) = Retention volume, eluted sample volume

V(SF) = Volume of displaced stationary phase

V(TOT) = Total coil volume

Determination of Solvent System

The favored solvents were added to a flask, shaken to reach saturation of upper and lower phase. Identical amounts of the crude extract were then added to equal amounts of upper and lower phase, respectively. After dissolving the sample in the two phases, equal amounts of the phases were analyzed by TLC on silica gel. After developing the plate with the organic layer of the solvent system once, the plate was sprayed with anisaldehyde/sulfuric acid reagent and heated. The spots on the TLC were compared and if the compounds showed an identical intensity in the upper and lower phase, optimal partition of the compounds between the phases was reached. The system n-hexane/ethylacetate/methanol/water (6:5:6:5, v/v/v/v) fulfilled the condition. Furthermore, R_f-values (retention factor) of the kavalactones were between 0.2 and 0.5, as recommended.[14]

HPLC-ESI-MS

The characterization of the extracts and isolated compounds was performed on a Bruker Esquire HPLC-MS driven by Bruker Daltonics software. The HPLC system consisted of a binary HP HPLC Pump G 1312 A with a Hitachi L-4000 UV Detector, which was set to 246 nm, and a Chromatopac C-R6A plotter. For the experiments a Prontosil 120 C18 AQ column (4.6 mm × 250 mm, 5.0 μm particle size) with a precolumn (5 mm × 4 mm) from Knauer (Berlin, Germany) was used. As mobile phase a water (solvent A) methanol (solvent B) gradient was chosen. The initial conditions were 90% A, 10% B; to 23% A, 77% B in 15 min; to 20% A, 80% B in 5 min; isocratic at 20% A, 80% B for 10 min; to 0% A, 100% B in 10 min and back to the initial conditions within 5 minutes. The flow rate was 0.5 mL/min.

The HPLC-ESI-MS parameters were the following: the spectra were recorded in the positive mode. Dry gas was nitrogen at a flow rate of 9.0 L/min and a temperature of 310°C. Nebulizer: 40.00 psi, capillary: 3500 V; end plate: −500 V; capillary exit: 94.6 V; skim 1:25.0 V, skim 2:6.0 V, fragmentation amplitude was 1.0 V.

The ESI-MS/MS experiments were carried out with the following parameters: dry gas (nitrogen) rate was 4.0 L/min, dry gas temperature: 300°C, nebulizer: 10.0 psi, capillary: 3500 V, capillary exit: 105.0 V, end plate: −500 V, skim 1:35.0 V, skim 2:10 V, and varying fragmentation amplitudes.

NMR

The experiments were carried out on a Bruker AMX 300 spectrometer (300 MHz for ^{1}H and 75 MHz for ^{13}C). The kavalactones were dissolved in chloroforme-d.

δ_H ppm for 5,6-dihydroyangonin: 2.53 (1H, dd, J = 4.4, J = 17.1 Hz, H-5_e), 2.66 (1H, ddd, J = 0.9, J = 10.6, J = 17.1 Hz, H-5_a), 3.76 (3H, s, OCH_3, H-15), 3.81 (3H, s, OCH_3, H-17), 5.03 (1H, ddd, J = 4.4, J = 7.0, J = 10.6 Hz, H-6), 5.19 (1H, d, J = 0.9 Hz, H-3), 6.12 (1H, dd, J = 6.8, J = 15.9 Hz, H-7), 6.67 (1H, d, J = 15.9 Hz, H-8), 6.86 (2H, ddd, J = 1.9, J = 2.8, J = 8.7 Hz, H-11/H-13), 7.33 (2H, ddd, J = 1.9, J = 2.8, J = 8.7 Hz, H-10/H-14). δ_C ppm for 5,6-dihydroyangonin: 33.81 (C-5, -CH_2-), 55.66 (OCH_3, C-17), 56.38 (OCH_3, C-15), 76.48 (C-6), 90.99 (C-3), 114.50 (2C, C-11/C-13), 123.69 (C-7), 128.34 (2C, C-10/C-14), 128.93 (C-9), 133.22 (C-8, quart. C), 160.23 (C-12, quart. C), 167.08 (C-2, quart. C), 172.65 (C-4, quart. C).

δ_H ppm for trans-yangonin: 3.82 (3H, s, OCH_3, H-15), 3.83 (3H, s, OCH_3, H-17), 5.47 (1H, d, J = 2 Hz, H-3), 5.89 (1H, d, J = 2 Hz, H-5), 6.44 (1H, d, J = 15.8 Hz, H-7), 6.90 (2H, ddd, J = 1.9, J = 2.8, J = 8.8 Hz, H-11/H-13), 7.44 (2H, ddd, J = 1.9, J = 2.8, J = 8.7 Hz, H-10/H-14), 7.46 (1H, d, J = 8.7 Hz, H-8).

RESULTS AND DISCUSSION

The kavalactones were supposed to be isolated from the crude extracts by HSCCC without preliminary purification. In a first step, a two phase solvent system had to be found which enables the isolation of the kavalactones in one run. For the determination of an optimal HSCCC solvent system it has to be taken into account that a partition coefficient of 1 is regarded as ideal.[15] As all of the major kavalactones were desired to be separated in one run, a solvent system was required, which would yield partition coefficients between 0.5 and 1. A partition coefficient much bigger than 1 would result in peak broadening and long separation times. Thus, higher amounts of solvents would be necessary. High sample capacity is only achieved if the extract dissolves equally well in the upper and lower phase.

Polar and non polar systems were tested. The solubility of the kavalactones soon turned out to be the limiting factor, because high ratios of n-hexane and water caused parts of the crude extract to precipitate. Saturation was reached whenever more water was used than methanol. Taking the required partition coefficients and the solubility of the sample into account, the solvent system n-hexane/ethyl acetate/methanol/water (6:5:6:5, v/v/v/v) emerged to be the most promising choice.

The ethanol extract (450 mg) was separated by HSCCC (Figure 2, HSCCC-1). After 7.5 h, the chromatographic separation was stopped; eight

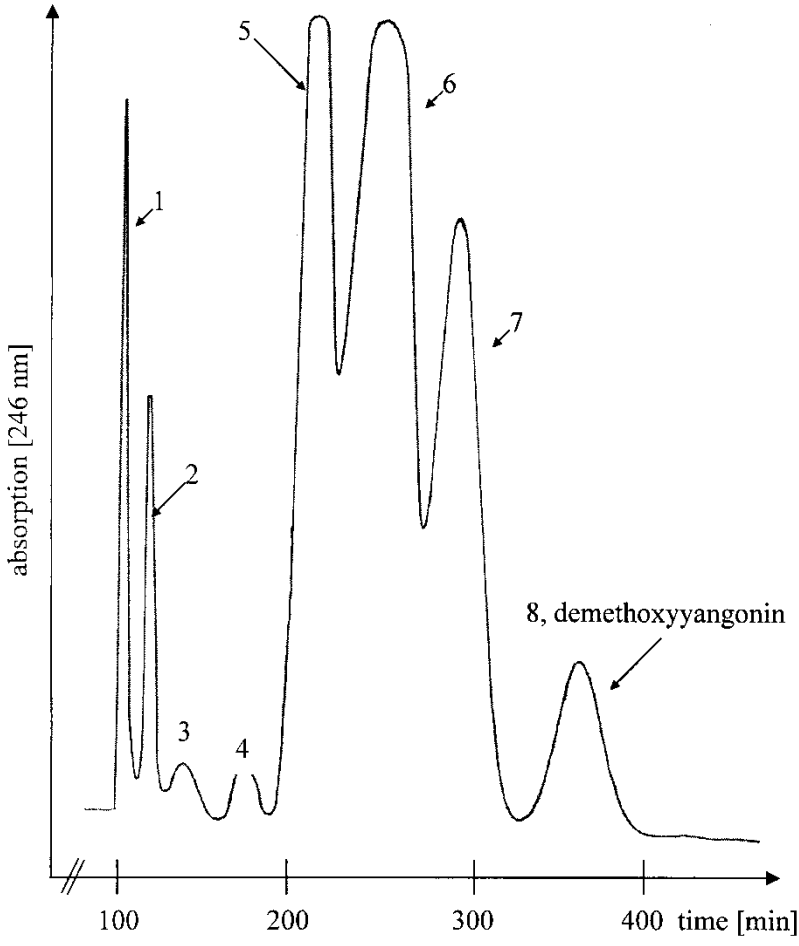

Figure 2. HSCCC separation of the ethanol crude extract showing fractions 1 through 8; fraction 8 contained demethoxyyangonin (HSCCC-1). Solvent system n-hexane/ethyl acetate/methanol/water (6:5:6:5, v/v/v/v), flow rate 3 mL/min, 1000 rpm (rotations per minute), head-to-tail modus.

fractions were collected and analyzed by HPLC-MS. Fractions 1 through 4 contained polymeric substances and minor kavalactones, whereas the major kavalactones were located in fractions 5 through 8. In fraction 5, methysticin, 7,8-dihydromethysticin, and 5,6,7,8-tetrahydroyangonin were detected. Because of insufficient resolution of peaks, 5 and 6, 7,8-dihydromethysticin and 5,6,7,8-tetrahydroyangonin were also present in fraction 6, besides kavain. In fraction 7 kavain was present together with yangonin and dihydrokavain. The last fraction contained only demethoxyyangonin. The retention of stationary phase was 67%.

In the next step, larger amounts of the crude extract (700 mg and 1000 mg) were injected. In both cases, peak resolution was poor. The major kavalactones, except for demethoxyyangonin, were present in one big fraction (chromatograms not shown). However, the minor lactones were separated.

In order to improve the retention of stationary phase and the quantity of injected sample, the crude extract was purified prior to HSCCC. This time, an acetone crude extract was used as it contained higher concentrations of kavalactones than the ethanolic extract. Gel chromatography on Sephadex LH-20 was applied to separate the polymeric material from the kavalactones and to concentrate the kavalactones. Altogether, six fractions were collected according to TLC results and analyzed by HPLC-MS. Only Fraction III was of importance, as it contained the main kavalactones.

Fraction III (820 mg) was subjected to a high-speed countercurrent chromatographic separation (Figure 3, HSCCC-2). This time, the retention of stationary phase was 84%. The obtained fractions were analyzed by HPLC-MS. Fractions 1 through 4 contained minor kavalactones. The partition coefficients of fractions 5 to 8 were calculated (Table 1) and ranged from 0.58 to 1.41. As the peak resolution was again not satisfactory for fractions 5 through 7, the twelve-milliliter fractions were also analyzed by TLC on silica gel, as all of the major kavalactones color differently with anisaldehyde/sulfuric acid spraying reagent after heating the plate. The twelve-milliliter fractions of peak 5 and 7 were combined according to the chromatogram as the compounds of peak 5 colored blue (R_f-value 0.28) and of peak 7 green (R_f-value 0.39) on the TLC plates. Peak 6 was separated into three subfractions, a through c. The compounds of fraction 6a turned into blue-green spots after spraying the TLC plate with the spraying reagent (R_f-value 0.29), fraction 6c turned bright red (R_f-value 0.36), and fraction 6b contained a mixture of the colors of 6a and 6c. HPLC-MS analysis enabled the characterization of the obtained fractions. Fraction 5 contained methysticin, fraction 6a 7,8-dihydromethysticin and 5,6,7,8-tetrahydroyangonin, fraction 6b kavain, 7,8-dihydromethysticin, and 5,6,7,8-tetrahydroyangonin, fraction 6c kavain, fraction 7 7,8-dihydrokavain and yangonin, and fraction 8 demethoxyyangonin.

The fractions were also analyzed by normal phase HPLC. Fractions 6c and 8 contained only one major compound. Kavain (76 mg) was obtained with 98% and demethoxyyangonin with 85% purity, respectively.

To isolate the remaining major kavalactones, the HSCCC fractions were further purified using preparative normal phase HPLC. To gain pure demethoxyyangonin, the isocratic system n-hexane/ethyl acetate (7:2, v/v) was used to remove small amounts of kavain. Fractions 6a and 7 were separated with the isocratic system n-hexane/p-dioxane (85:15, v/v) yielding 7,8-dihydromethysticin and 5,6,7,8-tetrahydroyangonin, 7,8-dihydrokavain and yangonin, respectively. After the analysis of fraction 5 with normal phase HPLC, another compound was detected besides methysticin, which was isolated with the isocratic system n-hexane/p-dioxane (85:15, v/v) (Figure 4).

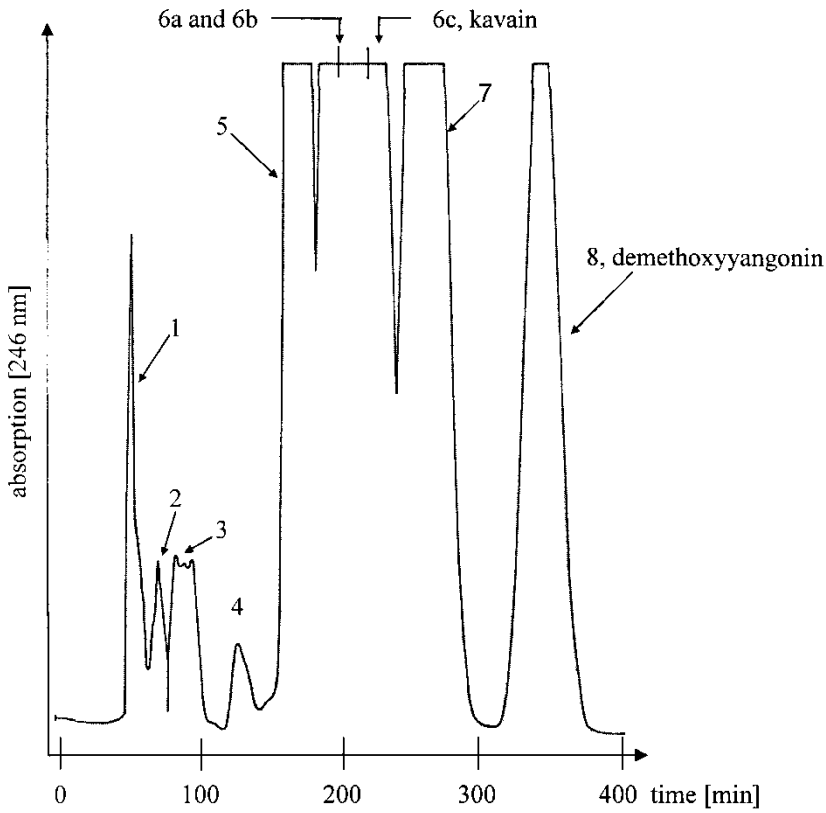

Figure 3. HSCCC separation of the purified acetone crude extract showing fractions 1 through 8; fraction 6c contained kavain and fraction 8 demethoxyyangonin (HSCCC-2). Solvent system n-hexane/ethyl acetate/methanol/water (6:5:6:5, v/v/v/v), flow rate 3 mL/min, 1000 rpm, head-to-tail modus.

Table 1. Partition coefficients of fractions 5 through 8 for the system n-hexane/ethyl acetate/methanol/water (6:5:6:5, v/v/v/v)

Fraction	Partition coefficient
5	0.58
6 a	0.68
6 b	0.77
6 c	0.87
7	1.03
8	1.41

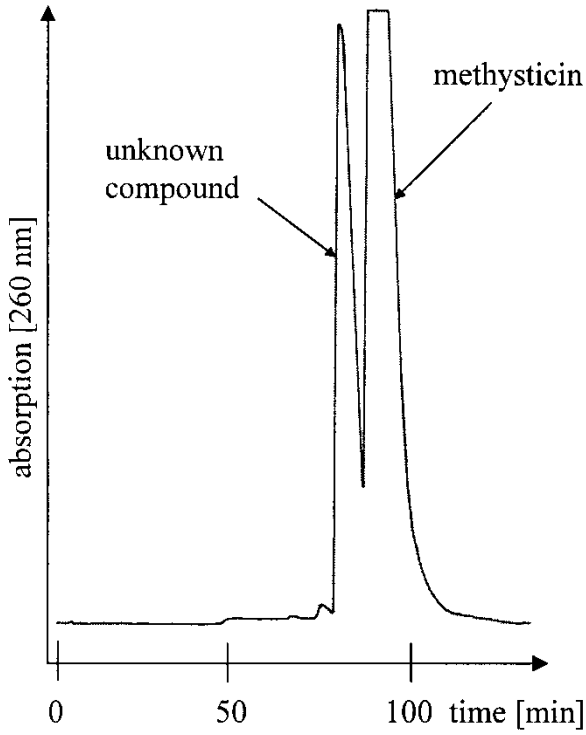

Figure 4. Preparative normal phase HPLC chromatogram of fraction 5 yielding the unknown compound and methysticin. Solvent system n-hexane/p-dioxane (85:15, v/v), flow rate 8 mL/min.

For all of the pure compounds structure elucidation was carried out using ESI-MS/MS and NMR spectroscopy. The obtained data was in agreement with literature data.[10,12] The unknown substance was identified as 5,6-dihydroyangonin (8) (Figure 1) and yangonin as the trans isomer.

The stability of trans-yangonin was monitored by HPLC, as Smith et al.[16] mentioned the cis-trans isomerization of yangonin to equilibrate after 90 min. The sample was dissolved in methanol and chloroform, respectively, and analyzed by HPLC-MS after 2 hours of incubation. Only one isomer was visible in the chromatogram of the isolated compound. The NMR spectra of the isolated yangonin showed only signals for the trans isomer. This investigation was carried out as stable standard compounds are necessary for biological tests.

Further TLC experiments were carried out to explain the coloration of the plates after spraying with anisaldehyde/sulfuric acid spraying reagent. Of interest were fractions 5, 6a, 7, which contained more than one major kavalactone. The pure compounds were analyzed by TLC. Methysticin and

5,6-dihydroyangonin emerged to have the same R_f-values (0.28) in the system n-hexane/ethyl acetate 1:1 (v/v), whereas methysticin colors blue and 5,6-dihydroyangonin blue-purple (Table 2). Surprisingly, 7,8-dihydromethysticin and 5,6,7,8-tetrahydroyangonin had identical R_f-values (0.29), and both substances colored blue-purple after spraying the TLC plate. 7,8-Dihydrokavain and trans-yangonin had the same R_f-values as well (0.39), but they could be differentiated by color. 7,8-Dihydrokavain yielded a brown-grey spot, whereas trans-yangonin gave a green spot.

CONCLUSION

It was shown that high-speed countercurrent chromatography can be used for the fractionation of crude kava kava extracts. However, it is almost impossible to separate all of the constituents in one run in general, especially if the extracts contain lipophilic and hydrophilic substances. In the case of kava kava, good separation was achieved only with a relative small amount of sample.

To improve the retention of stationary phase and, therefore, to potentiate the input of sample, the polymeric substances of the crude extract were partially removed by gel chromatography on Sephadex LH-20 material. At the same time the kavalactones were concentrated. The HSCCC chromatogram of the purified acetone extract showed an improvement of the retention of stationary phase, whereas the peak resolution between fractions 5 and 7 was not enhanced.

Table 2. R_f-values (retention factor) and characteristic color of the standard compounds after spraying the TLC (silica gel) plate with anisaldehyde/sulfuric acid spraying reagent and heating. Solvent system n-hexane/ethyl acetate (1:1, v/v), developed once

	R_f-value	Color after spraying with spray reagent
Methysticin	0.28	blue
5,6-dihyrogyangonin	0.28	blue-purple
7,8-dihydromethysticin	0.29	blue-purple
5,6,7,8-tetrahydroyangonin	0.29	blue-purple
Kavain	0.38	red
7,8-dihydrokavain	0.39	brown-grey
trans-yangonin	0.39	directly after heating blue, then color turns green
Demethoxyyangonin	0.42	pink-purple, color diminishes after a while

Although it was aimed to isolate the six major kavalactones with HSCCC in a single run, only two of the main compounds were obtained that way, kavain with 98% purity and demethoxyyangonin with 85% purity. The remaining main kavalactones had to be additionally purified by preparative HPLC. Because of the encountered difficulties, further studies with TLC on silica gel and normal phase HPLC were required.

ABBREVIATIONS

HSCCC	high-speed countercurrent chromatography
HPCPC	high performance centrifugal partition chromatography
V (RT)	retention volume
V (SF)	volume of displaced stationary phase
V (TOT)	total coil volume
R_f	retention factor.

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