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Journal of Chromatography A, 825 (1998) 1–8

JOURNAL OF CHROMATOGRAPHY A

Reversed-phase high-performance liquid chromatographic method for quantitative analysis of the six major kavalactones in *Piper methysticum*

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Received 10 March 1998; received in revised form 6 August 1998; accepted 24 August 1998

Abstract

A reversed-phase high-performance liquid chromatographic method to simultaneously measure the six major kavalactones [methysticin, dihydromethysticin (DHM), kavain, dihydrokavain (DHK), demethoxyyangonin (DMY) and yangonin] in *Piper methysticum* (kava) has been successfully developed. The method uses the six compounds as external standards. These compounds were isolated in our laboratory using various chromatographic methods. The six compounds are completely separated within 35 min using a YMCbasic S-5 column and an isocratic methanol–acetonitrile–water–acetic acid (20:20:60:0.1, v/v) mobile phase at 40°C. The quantitative calibration curves are linear covering a range of 5–500 μg/ml for all six compounds. The detection limits ($S/N=3$) for methysticin, DHM, kavain, DHK, DMY and yangonin are approximately 0.5, 1.1, 0.7, 1.1, 0.6 and 0.6 μg/ml, respectively. The average recoveries are 100.2% for methysticin, 100.6% for DHM, 100.0% for kavain, 100.3% for DHK, 98.9 for DMY and 98.2% for yangonin with R.S.D.s less than 3.6%. The six peaks in the HPLC chromatogram of kava extract were confirmed by LC–atmospheric pressure positive chemical ionization MS and their purities were examined by on-line UV and mass spectral analyses. In order to simplify the determination of the total kavalactone level, response factors and correlation factors relative to kavain were established.

Keywords: *Piper methysticum*; Kava; Kavalactones; Methysticin; Kavain; Yangonin; Dihydrokavain; Dihydromethysticin; Demethoxyyangonin

1. Introduction

Piper methysticum, popularly known as kava, is widely distributed through the south Pacific, from Hawaii to New Guinea [1]. In the daily life of the Pacific island people, the root is used to prepare a drink for important social and ceremonial occasions, similar to the use of coffee or tea in other cultures. The root is also used by local people for health purposes such as soothing the nerves, inducing relaxation and sleep, counteracting fatigue and reducing mass [2,3]. In addition to the local uses, kava has long been utilized in European phytomedicine as a sedative, tranquilizer, muscle relaxant, treatment for menopausal symptoms and treatment for urinary tract and bladder disorders [4]. A large number of compounds have been isolated from this plant over the past hundred years in the search for the biologically active constituents [5–17]. Most of these

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0021-9673/98/$ – see front matter
PII: S0021-9673(98)00699-2

compounds are kavalactones which can be classified as substituted α-pyrones or substituted 5,6-dihydro-α-pyrones. Many pharmacological studies on the purified compounds from the roots suggested that the biological activity of the plant is mainly attributable to the kavalactones [18–29]. Among the kavalactones, the six compounds, methysticin, dihydromethysticin (DHM), kavain, dihydrokavain (DHK), demethoxyyangonin (DMY) and yangonin (Fig. 1) account for ca. 98% [30]. The quantity of these six compounds is therefore used as a quality control measure in standardized phytotherapeutical and neutraceutical preparations.

Many attempts have been made to separate the kavalactones by thin-layer chromatography (TLC), gas chromatography (GC), gas–liquid chromatography (GLC) or high-performance liquid chromatography (HPLC) methods, beginning with the pioneering work of Nakayama et al., who quantitatively measured the six compounds by TLC in combination with UV spectroscopy [31]. However, the TLC method was not precise or demanding, and the average recoveries were only 80–95%. In a GC method, several kavalactones were quantified [32]. A GLC method was used for quantitative determination of the major kavalactones, but methysticin and yangonin which are two of the major components could not be separated. In addition, the high temperature of the injection port caused the decomposition of methysticin [33]. In a normal-phase HPLC method, the five kavalacones were successfully separated and quantified, but DMY was not included [34]. Very recently, Häberlein et al. developed a normal-phase HPLC method on a ChiraSpher NT column which successfully separated the six compounds [35]. In the meantime, they reported the quantitative determination of kavapyrones in kava sample using the same HPLC method [36]. A reversed-phase HPLC method using an ODS- Hypersil column and methanol–water (55:45) as the mobile phase was reported by Smith et al., but the overall separation was found to be very poor [37]. More recently, reversed-phase HPLC–electrospray ionization (ESI) mass spectrometry (MS) was used to identify the constituents in kava extract [38]. The HPLC chromatogram showed that DHM and kavain were poorly separated.

In spite of few difficulties associated with the normal-phase resolution of all components, the use of undesirable toxic and volatile organic solvents and the requirement of long equilibration times have posed problems for the utility of these analytical methods. Furthermore, sample preparation for normal-phase column chromatography is complicated by the fact that the injection sample must be free of all traces of water. The steadily increasing number of kava samples needing analysis requires a faster, more convenient assay method. Therefore, the need for a reversed-phase HPLC method which is favored in modern analytical methods became evident.

The oily properties of kava extract and the structural similarities of kavalactones make the separation by reversed-phase HPLC very challenging. A large number of tests using various stationary phases and mobile phases were carried out. Eventually, a method using a YMCbasic S-5 column with methanol–acetonitrile–water–acetic acid as a mobile phase was developed which enables the six major kavalactones to be completely separated.

In order to confirm the identity of the individual peaks in the HPLC chromatogram of the kava extract, LC–atmospheric pressure chemical ionization in the positive mode [(+)-APCI] MS analyses were undertaken.

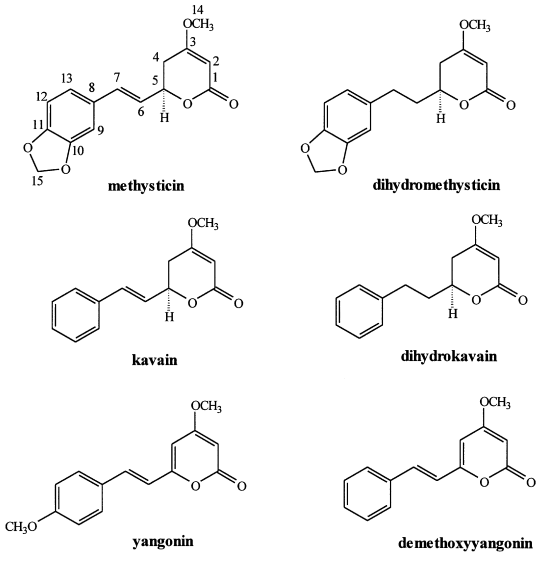

Fig. 1. Structures of the six major kavalactones in *Piper methysticum* (kava).

2. Experimental

2.1. Solvents and reagents

HPLC-grade acetonitrile, methanol and water were purchased from Fisher Scientific (Fair Lawn, NJ, USA). HPLC-grade hexane, 1,4-dioxane, ethyl acetate and acetone were purchased from Sigma (St. Louis, MO, USA). HPLC-grade acetic acid was purchased from J.T. Baker (Phillipsburg, NJ, USA). Silica gel (130–270 mesh, 60 Å) for open column chromatography was purchased from Aldrich (Milwaukee, WI, USA). The six standards, methysticin, DHM, kavain, DHK, DMY and yangonin were isolated from the roots of *Piper methysticum* using chromatographic methods described below.

2.2. Isolation of six major kavalactones from the kava extract

The roots of *P. methysticum* (60 g) were extracted with 95% ethanol three times at 40°C (each 600 ml, 24 h). The combined extract was concentrated by rotary evaporation in vacuo at 40°C to dryness (20 g). The residue was loaded onto a silica gel column (45.7×3.8 cm, 200 g, 130–270 mesh) eluted with a hexane–EtOAc gradient [(4:1) to (2:1), total volume 10 l], and 18 fractions were collected. The fifth fraction contained mostly DMY and was subjected to column chromatography over silica gel (60 g) eluting with hexane–acetone (4:1, 1.2 l) to give 85 mg of DMY as colorless needles. The eighth fraction contained DHK and DMY and was initially chromatographed over silica gel (80 g) using hexane–acetone (3:1) as the mobile phase. The fraction which mainly contained DHK was subjected to separation by preparative HPLC (column: Dynamax-60A silica gel, 25×2.14 cm, 8 μm) using hexane–dioxane (4:1) as the mobile phase with a flow-rate of 10 ml/min to give 51 mg of DHK as colorless needles. Fraction 10 which contained mostly kavain was recrystallized with methanol to yield 160 mg of kavain as colorless needles. Fractions 13 and 14 were combined and purified by preparative HPLC using hexane–dioxane (3:1) as the mobile phase to yield 50 mg of DHM as colorless needles. Fractions 15 and 16 were combined and subjected to column chromatography over silica gel to give 450 mg of yangonin as light yellow needles. Fractions 17 and 18 were combined and repeatedly chromatographed over silica gel, and further purified by recrystallization to yield 1.416 g of methysticin. Preparative HPLC was performed on a Dynamax HPLC system (Varian, Walnut Creek, CA, USA) equipped with a dual pump system, a UV–Vis absorbance detector, and a FC-1 fraction collector. These compounds were identified by comparing the UV, IR and MS spectral data with the literature data [39]. Their structures were confirmed by ^{1}H nuclear magnetic resonance (NMR) data which were obtained on a Varian 400 MHz NMR instrument. ^{1}H NMR ($C^{2}HCl_3$) δ_H ppm: for methysticin: 2.53 (1H, dd, J=17.0, 4.4 Hz, H-4α), 2.66 (1H, dd, J=17.0, 12.0, H-4β), 3.77 (3H, s, H-14), 5.03 (1H, ddd, J=17.0, 4.4, 6.4 Hz, H-5), 5.20 (1H, s, H-2), 5.97 (2H, s, H-15), 6.09 (1H, dd, J=16.0, 6.4 Hz), 6.64 (1H, d, J=16.0 Hz, H-7), 6.77 (1H, d, J=8.0, 2.0 Hz, H-13), 6.84 (1H, d, J=8.0 Hz, H-12), and 6.93 (1H, d, J=2.0 Hz, H- 9); for dihydromethysticin: 1.86 (1H, m, H-6a), 2.07 (1H, m, H-6b), 2.28 (1H, dd, J=17.0, 4.4 Hz, H-4α), 2.49 (1H, dd, J=17.0, 12.0 Hz, H-4β), 2.73 (1H, m, H-7a), 2.79 (1H, m, H-7b), 4.34 (1H, m, H-5), 5.13 (1H, s, H-2), 5.91 (2H, s, H-15), 6.64 (1H, dd, J=8.0 Hz, H-12), 6.68 (1H, d, J=2.0 Hz, H-9), and 6.72 (1H, dd, J=8.0, 2.0 Hz, H-13); for kavain: 2.55 (1H, dd, J=17.0, 4.4 Hz, H-4α), 2.68 (1H, dd, J=17.0, 12.0 Hz), 3.78 (3H, s, H-14), 5.08 (1H, ddd, J=17.0, 4.4, 6.0 Hz, H-5), 5.21 (1H, s, H-2), 6.27 (1H, dd, J=15.2, 6.0 Hz, H-6), 6.74 (1H, J=15.2 Hz, H-7), and 7.29–7.41 (5H, m, H-9–H-13); for dihydrokavain: 1.94 (1H, m, H-6a), 2.14 (1H, m, H-6b), 2.30 (1H, dd, J=17.0, 4.4 Hz, H-4α), 2.51 (1H, dd, J=17.0, 12.0 Hz, H-4β), 2.80 (1H, m, H-8a), 2.89 (1H, m, H-8b), 3.74 (3H, s, H-14), 4.37 (1H, m, H-5), 5.15 (1H, s, H-2), and 7.21–7.30 (5H, m, H-9–H-13); for yangonin: 3.83 and 3.84 (each 3H, s, H-14, H-15), 5.48 (1H, d, J=1.6 Hz, H-2), 5.90 (1H, d, J=1.6 Hz, H-4), 6.45 (1H, d, J=16.0 Hz, H-6), 6.91 (2H, d, J=8.0 Hz, H-10, H-12), 7.46 (2H, d, J=8.0 Hz, H-9, H-13), and 7.47 (1H, d, J=16.0 Hz, H-7); for demethoxyyangonin: 3.83 (3H, s, H-14), 5.01 (1H, d, J=1.6 Hz, H-2), 5.96 (1H, d, J=1.6 Hz, H-4), 6.59 (1H, d, J=16.0 Hz, H-6), and 7.34–7.54 (6H, m, H-7, H-9–H-13). The purities were checked by HPLC coupled with a UV–Vis detector and a mass spectrometer.

2.3. HPLC

Analytical HPLC analyses were performed on a Hewlett-Packard 1100 modular LC system equipped with an autosampler, a quaternary pump system, a photodiode array detector, a thermostatted column compartment and a HP chemstation data system. The chromatographic separations were carried out using a 25 cm×4.6 mm I.D. YMCbasic reversed-phase analytical column, 5 μm particle size (YMC, Wilmington, NC, USA). An isocratic mobile phase of methanol–acetonitrile–water–acetic acid (20:20:60:0.1, v/v) was used with a flow-rate of 1 ml/min. The absorption spectra were recorded from 200 to 400 nm for all peaks. Quantitation was carried out at a single wavelength of 220 nm. The column was thermostatted at 40°C.

2.4. LC–MS

The HP 1100 LC system was coupled with a Finnigan LCQ mass spectrometer (Finnigan MAT, San Jose, CA, USA). The mass spectrometer was operated in a continuous scanning mode over a mass range of 50–500 u using an (+)-APCI probe. The APCI source parameters were optimized by continuous injection of standards scanned in a select ion monitor mode. The vaporizer and capillary temperatures were 450 and 150°C, respectively. The HPLC fluid was nebulized using N_2 as both a sheath gas and an auxiliary gas at flow-rates of 80 and 10 arbitrary units, respectively. The discharge current, discharge voltage, capillary voltage and tube lens offset were 5.00 μA, 3.99 kV, 3.00 V and −15.0 V, respectively.

2.5. Sample preparation

Five grams of ground kava roots were extracted overnight with 180 ml of 95% ethanol in a shaker. After filtration, the solution was brought to a volume of 200 ml with the same solvent. This sample was filtered through a 0.45-μm membrane and directly subjected to HPLC analysis.

2.6. Preparation of mixed standard solution and calibration

The six kavalactones, methysticin (5.1 mg), DHM (5.0 mg), kavain (6.0 mg), DHK (5.6 mg), DMY (5.0 mg) and yangonin (5.0 mg) were dissolved in methanol and brought to a volume of 10 ml. Calibration curves were established based on seven data points covering a concentration range of 5.1–510 μg/ml for methysticin, 5.0–500 μg/ml for DHM, 6.0– 600 μg/ml for kavain, 5.6–560 μg/ml for DHK, 5.0–500 μg/ml for DMY and 5.0–500 μg/ml for yangonin. Five microliter aliquots were used for HPLC injections (n=5). The recoveries were checked by spiking three different known amounts of standard with a known amount of kava sample with low kavalactones content and the kavalactones content were requantitated.

2.7. Calculation of response factors and correlation factors relative to kavain

Response factors were calculated by the peak area (mAU) divided by the corresponding concentration (μg/ml) of the compound. Correlation factors were calculated by the response factor of each compound divided by the response factor of kavain.

3. Results and discussion

3.1. Development of the HPLC method

A wide variety of solvents were tested for their ability to separate the kavalactones present in the plant extract. Gradient and isocratic systems of methanol–water or acetonitrile–water in combination with acetic acid, phosphate buffer, phosphoric acid and trifluoroacetic acid on different stationary phases (RP-18, RP-8, phenyl and YMCbasic) did not result in the complete separation of the six major compounds. Eventually, the acetonitrile–methanol–water (20:20:60) isocratic system was used to achieve complete separation on a YMCbasic S-5 column. By adding 0.1% acetic acid, the resolution and sensitivity were improved. The ratio of acetonitrile, methanol and water was found to be very critical.

The column temperature was optimized by comparing the HPLC chromatograms obtained at 25°C, 40°C and 50°C. The temperature of the column oven slightly affects the resolution, and markedly affected the retention times of all six components. The resolution of DMY and yangonin decreased at 50°C

compared with that seen at 40°C. At 25°C, the run time was relatively long. Thus, a setting of 40°C was selected as the final column temperature.

The sensitivity of the analysis was examined by comparing the peak response of the six kavalactones at a wavelength of 220 and 240 nm. The response of DHM, kavain, and DHK was stronger at 240 nm, but that of methysticin, DMY and yangonin were stronger at 220 nm. Because the kavain content is usually highest in kava samples, we selected 220 nm as the appropriate wavelength for detection and quantitation.

3.2. Characterization of six major kavalactones in kava extract by LC–(+)-APCI-MS

For the LC–APCI-MS analysis of the extract, the chromatographic conditions were the same as those described for the HPLC analysis. On-line APCI mass spectra were recorded in the positive ion mode. Fig. 2 shows the on-line HPLC–UV (A) and total ion (B) chromatograms, and the mass spectra corresponding to the six major peaks (C–H). The APCI mass spectra of the six compounds gave base peaks due to the cationized molecular ions $[M+H]^+$. The molecular masses obtained confirmed the identification of the six compounds on the basis of comparison of the retention time (t_R) and UV absorption with isolated samples. The purity of each peak was greater than 99%, as calculated from the on-line UV spectrum. Mass spectral data further showed that no extraneous peaks interfered with the six peaks, since no other ion peaks except for the molecular ion peaks appeared markedly in the mass spectra.

3.3. Calibration

Calibration curves for the six kavalactones were established as described in Section 2.6. They showed excellent linearity covering the range of ca. 5.0–600.0 μg/ml (Table 1), with correlation coefficients in the range 0.99998–1. In addition, the curves remained consistent during the working life of the column (at least 1000 injections), making it unnecessary to construct a new calibration curve each day. The calibration is normally checked by running a triplicate standard injection prior to injection of the unknown sample. Recalibration is considered only if the mean difference between the known standard and the calculated values exceeds 5%.

3.4. Recovery

The recoveries were determined by adding various quantities of the standards in the ranges of 10.2–163.2 μg/ml for methysticin, 10.0–160.0 μg/ml for DHM, 12.0–198.0 μg/ml for kavain, 11.2–179.2 μg/ml for DHK, 10.0–160.0 μg/ml for DMY, and 10.0–160.0 μg/ml for yangonin to kava root extracts and repeating the analyses ($n=5$). The results are listed in Table 2. The average recoveries were 100.2% for methysticin, 100.6% for DHM, 100.0% for kavain, 100.3% for DHK, 98.9 for DMY and 98.2% for yangonin.

3.5. Detection limit and precision

The detection limit was measured as the concentration corresponding to a signal-to-noise ratio of 3:1. According to this rule, the values for methysticin, DHM, kavain, DHK, DMY and yangonin were approximately 0.5, 1.1, 0.7, 1.1, 0.6 and 0.6 μg/ml, respectively. Precision was evaluated by performing five replicate analyses of the kava extract within the same working day. The average relative standard deviations (R.S.D.s) for the six compounds were less than 3.5%, suggesting that the method has excellent precision.

3.6. Response factors and correlation factors

Among the six kavalactones, kavain is the only one that is commercially available. Therefore, it is helpful for industrial quality control purpose to develop a calibration method using kavain as an external standard to determine the total kavalactones in kava sample. To simplify the calibration method, the response factors at various concentrations were studied. Calibration equations for the six kavalactones were used to calculate the responses (peak area) at five different concentrations (40, 80, 160, 320 and 500 μg/ml), and the response factors were then calculated. The average response factor for methysticin, DHM, kavain, DHK, yangonin and DMY was 21.96, 9.52, 15.75, 8.59, 18.80 and 19.86, respectively. The R.S.D.s of the values are less than 1.27%. The correlation factor relative to kavain for

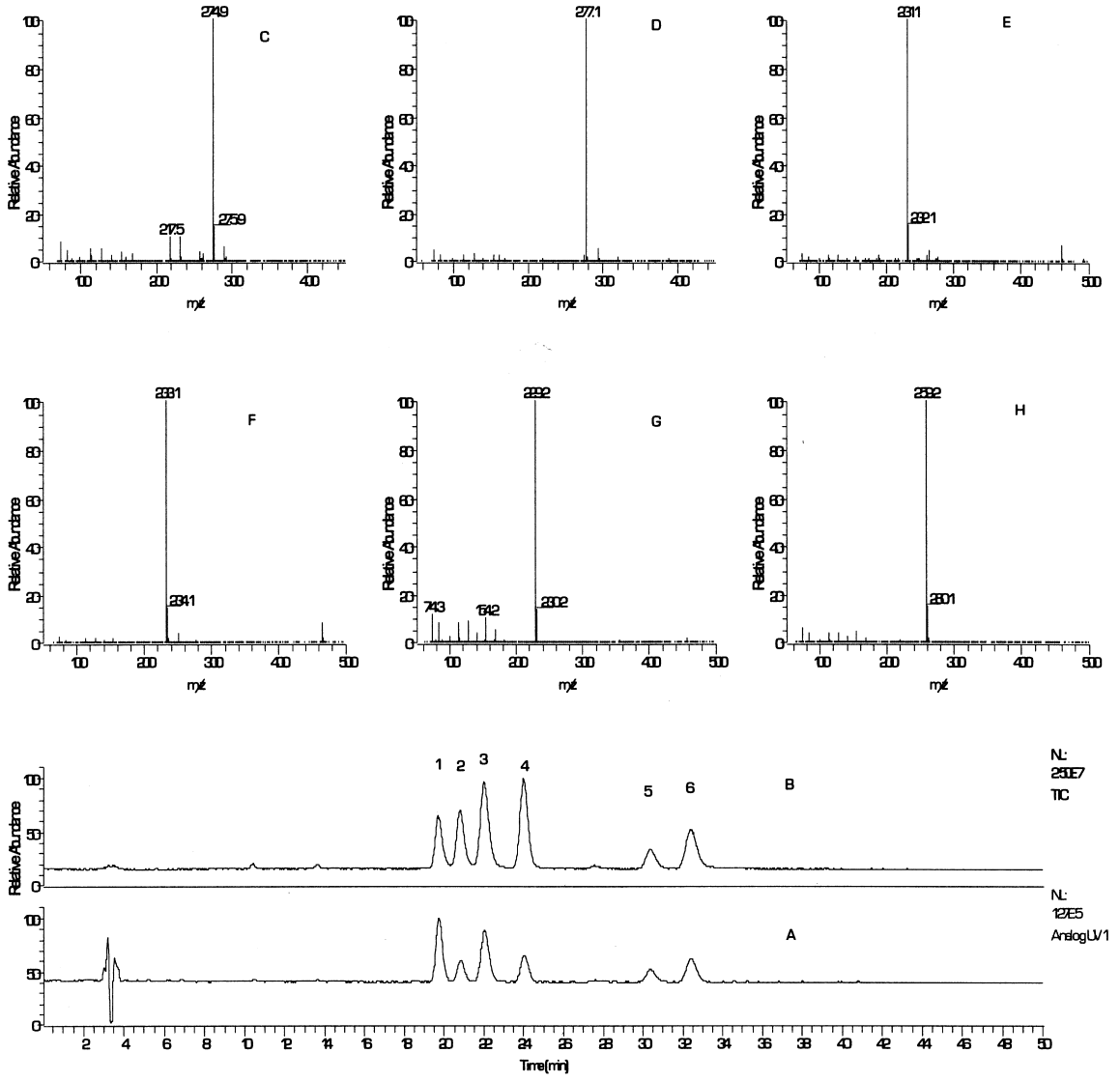

Fig. 2. On-line LC–(+)-APCI-MS data of a kava root extract. (A) UV trace; (B) total ion trace; (C) MS spectrum of peak 1(methysticin); (D) MS spectrum of peak 2 (DHM); (E) MS spectrum of peak 3 (kavain); (F) MS spectrum of peak 4 (DHK); (G) MS spectrum of peak 5 (DMY); (H) MS spectrum of peak 6 (yangonin).

Table 1
Calibration data for six kavalactones with the present HPLC methods[a]

Compounds	Concentration range (μg/ml)	Slope	Intercept	Correlation coefficient
Methysticin	5.1–510.0	22.071	−11.683	0.99999
DHM	5.0–500.0	9.5153	0.339	1
Kavain	6.0–600.0	15.757	−0.491	1
DHK	5.6–560.0	8.5395	4.662	1
DMY	5.0–500.0	18.772	3.109	0.99998
Yangonin	5.0–500.0	20.118	−26.715	0.99999

[a] Regression analysis was performed according to the equation; y (peak area)$=m$ (slope)x (concentration, μg/ml)$+C$ (intercept).

Table 2
Recoveries of the six major kavalactones from the kava root extract[a]

Compound	Added (μg/ml)	Detected±S.D. (μg/ml)	Recovery (%)	R.S.D. (%)
Methysticin	10.2	10.3±0.3	101.0	2.9
	40.8	40.7±0.5	99.8	1.2
	163.2	163.0±1.0	99.9	0.6
DHM	10.0	10.1±0.3	101.0	3.0
	40.0	40.2±0.6	100.5	1.5
	160.0	160.3±1.2	100.2	0.8
Kavain	12.0	12.0±0.3	100.0	2.5
	48.0	47.8±0.5	99.6	1.0
	198.0	198.8±0.9	100.4	0.5
DHK	11.2	11.3±0.4	100.9	3.6
	44.8	44.9±0.5	100.2	1.1
	179.2	179.0±0.2	99.9	0.1
DMY	10.0	9.8±0.3	98.0	3.0
	40.0	39.7±0.6	99.3	1.5
	160.0	159.0±0.3	99.4	0.2
Yangonin	10.0	9.8±0.3	98.0	3.0
	40.0	39.3±0.7	98.3	1.8
	160.0	157.2±0.4	98.3	0.3

[a] Quintuplicate analyses.

methysticin, DHM, DHK, yangonin and DMY was determined to be 1.39, 0.60, 0.55, 1.19 and 1.26, respectively. The calibration method based on correlation factors was evaluated by analyzing a kava powdered extract. From the analysis, the total kavalactones was 30.7%, calculated as the six standards, and 30.8%, calculated as kavain. Therefore, the simplified calibration method based on the correlation factors is valid for the quantitative determination of the total kavalactones in kava sample.

4. Conclusions

The HPLC method described in this paper provides baseline separation of all six major kavalactones. It allows us to determine the quantity of the six kavalactones rapidly and accurately both in plant extracts and in preparations using a single run. The method uses an isocratic mobile phase that is very favorable since results are easily reproduced and the necessity of reequilibrating the column between injections is eliminated. The reversed-phase HPLC solvent systems are more environmentally acceptable than those used in normal-phase HPLC. The method also offers excellent reproducibility and high recoveries of the all analytes. Hence, the method is recommended for quality control analysis of kava samples.

The LC–(+)-APCI-MS technique was successfully applied for the identification of the six major kavalactones in a kava root extract. It used the same LC conditions as those employed in the standard reversed-phase HPLC analysis. This study demonstrates that the combination of HPLC and APCI-MS techniques is invaluable for the on-line analysis of natural products in plant extracts.

Acknowledgements

We gratefully thank Ms. Lynn Pilkington for her advice in the preparation of this manuscript, the Quality Control Department for experimental assistance, and the Production Department for provision of raw material, native extract and powdered extract.

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