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Review

Analysis of kavalactones from *Piper methysticum* (kava-kava)

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Abstract

The chemical analysis and quality control of both *Piper methysticum* G. Forster (kava-kava) and extracts obtained by aqueous acetone or aqueous methanol as well as supercritical fluid extraction are reviewed. In the last two decades various procedures concerning the separation and detection of kavalactones have been routinely carried out by gas chromatography (without previous derivatization of kavalactones) and high performance liquid chromatography but most of them are not validated or only partially validated. Recently, analyses by supercritical fluid chromatography and micellar electrokinetic chromatography have also been reported. Both gas chromatography and high performance liquid chromatography can be used for the analysis of kavalactones with some advantages and disadvantages for each method. Using gas chromatography analysis, methysticin and yangonin, which are two of the major components, are generally not separated. In addition, the high temperature of the injection port caused the decomposition of methysticin.Concerning high performance liquid chromatography analyses, the reversed-phase is generally better because highly reproducible with a very low detection limit for all compounds even if the quantitative analysis of the kavalactones by liquid chromatography needs to be carried out in the absence of light to prevent the *cis*/*trans* isomerisation of yangonin.

Keywords: *Piper methysticum* G. Forster; Kavalactones

Contents

1. Introduction

Kava-kava is the name given by Pacific islanders to both a shrub *Piper methysticum* G. Forster belonging to the pepper family and the psychoactive beverage made from the rhizome [1].

The first description of the plant was by two Swedish botanists during the first expedition of Captain James Cook in the South Pacific area (Endeaviour) in 1768–1771. On occasion of Captain James Cook's second voyage (1772–1775)

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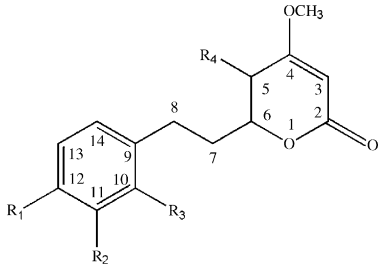
the botanist Johann Georg Forster named the plant *P. methysticum* meaning “intoxicating pepper” and gave the first detailed description of the plant. Thus, “methysticum” is the Latin transcription of the Greek “methustikos” and it is derived from “methu” which means “intoxicating drink” [2].

Kava-kava may have first been domesticated less than 3000 years ago in Vanuatu (nowadays called the New Hebrides), a group of islands in eastern Melanesia. The use of kava-kava seems then to have spread both westwards to New Guinea and part of Micronesia and eastward into Fiji and then Polynesia. Locally the plant is known by a number of common names, including kawa-kawa, ava ava, awa awa, yati, yagona, and yangona. In addition, the different varieties of this plant are reported with various vernacular names by indigenous populations such as Apu, Makea, Liwa, Mo’i, Papa [2]. The beverage is obtained from the rhizome by extraction with coconut milk [1,3] and it is used in social and ceremonial life because of the narcotic and soporific effects and as it counteracts fatigue, reduces anxiety and generate state of well being [1,2]. The plant has been used in the traditional medicine to treat both acute and chronic gonorrhea, vaginitis, leucorrhea, menstrual problems, venereal diseases, nocturnal incontinence and other ailments of the genitourinary tract as it has an antiseptic effect on urine [4]. Kava-kava rhizome was first introduced in 1914 in the British Pharmacopoeia and it appeared in the US Dispensary in 1950 [5]. The herbal drug is represented by the dried rhizome, usually free from roots and, sometimes, scraped. It contains not less than 3.5% kavalactones calculated as kavain [1]. Extracts of kava-kava with 30–70% kavalactones have also been employed in the western medicine for the sedative, muscle relaxant, analgesic, anticonvulsive, anaesthetic, anti-arrhythmic, anti-thrombosis, neuroprotective and anti-spasmodic effects [5].

The aim of this review is to give an overview of the separation procedures for kavalactones from the herbal drug or preparations, both qualitatively and quantitatively.



Kavalactones	R_1	R_2	R_3	R_4	C5-C6	C7-C8
11-Hydroxy-12-methoxydihydrokavain	OCH_3	OH				
7,8-Dihydro-5-hydroxykavain				β-OH		
11,12-Dimethoxydihydrokavain	OCH_3	OCH_3				
Methysticin	OCH_2O					≡
Dihydromethysticin	OCH_2O					
Kavain						=
7,8-Dihydrokavain						
5,6-Dehydromethysticin	OCH_2O				=	≡
5,6-Dehydrokavain (demethoxyyangonin)					=	=
Yangonin	OCH_3				=	≡
5,6,7,8-Tetrahydroyangonin	OCH_3					
5,6-Dihydroyangonin	OCH_3					≡
7,8-Dihydroyangonin	OCH_3				=	
10-Methoxyyangonin	OCH_3		OCH_3		=	≡
11-Methoxyyangonin	OCH_3	OCH_3			=	=
11-Hydroxyyangonin	OCH_3	OH			=	≡
Hydroxykavain				OH		=
11-Methoxy-12-hydroxydehydrokavain	OH	OCH_3			=	≡

Chalcones	R
Flavokavain A	OCH_3
Flavokavain B	H
Flavokavain C	OH

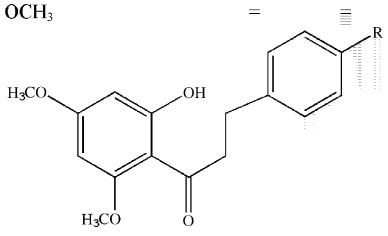


Fig. 1. Kavalactones and chalcones present in kava-kava rhizome.

2. Chemistry

The constituents responsible for the pharmacological activity of the herbal drug are a mixture of structurally related lipophilic lactones with an α-pyrone skeleton typically 4-methoxy-2-pyrones with aromatic stiryl or phenylethyl substituents at the 6-position and generally called kavalactones [6,10–14]. The first studies concerning their structure elucidation go back to the second half of the 19th century [15] and nowadays almost 20 different derivatives have been isolated and identified. The six major kavalactones account for 96% of the lipid extract and are dehydrokavain (demethoxyyangonin) (DEHK), dihydrokavain (DIHK), yangonin (Y), kavain (K), dihydromethysticin (DIHM) and methysticin (M) [7–9,16]. Their structures are reported in Fig. 1. Minor constituents include other kavalactones and three chalcones [7]. Essential oil and traces of piperidine alkaloids are also present [8].

The content of kavalactones in the dried rhizome depends on the age of the plant and the specific cultivar. Kavalactones are also present in other parts of the plant and their variability is also strongly affected by the geographical location of the cultivation.

Kavalactone content decreases progressively from roots to leaves [17] and there is significant variation in chemical composition according to the organ analysed [18–20]. DIHM and DIHK are the major components of the leaves and K and M are the major components of the roots and rhizome [19].

3. Chromatographic methods

Most phytochemical analyses of kava-kava herbal drug, extracts or preparations have focused on the kavalactones, considered as the active compounds of the herbal drug and herbal drug preparations. A number of methods have been developed for the determination of the major kavalactone constituents using thin-layer chromatography (TLC) and spectroscopic methods [21–24]. The pioneering work was in 1966 and the authors determined the amounts of the principal kavapyrones in the rhizome of six cultivars from Hawaii using two-dimensional thin-layer chromatography on aluminium oxide. They quantitatively measured the six major compounds by TLC in combination with UV spectroscopy [25]. However, this method was neither precise nor demanding, and the average recoveries were only 80–95%. Similar methods of analysis have been performed on kava-kava extracts and preparations by Coclers et al. (to estimate kavain) and Di Renzo (to estimate K, M and Y) [23,24]. Finally, a colorimetric method to determine the amounts of K, M and Y in extracts of rhizomes was used by Csupor [22]. In the early 1970s GLC and HPLC were also developed using different detection methods in order for the qualitative as well as quantitative analysis of kavalactones.

3.1. Gas chromatography

Gas–liquid chromatography (GLC) was first used in 1971 as a qualitative method for the analysis of constituents of kava-kava roots and led to the identification by MS detection of other constituents in addition to the known six major kavalactones [26]. The authors used a glass column containing silicone SE 30 and Chromosorb W 60/80. The temperature was programmed between 10 and 300 °C with increasing steps of 15 °C min^{-1}.

A decade later, GLC analyses were applied to quantitative evaluation of kavalactones in the roots and rhizome [27] and of kavalactones and alkaloids in different plant parts [18,28]. In 1981 Duve performed analysis on a glass column containing 3% m/m of OV-1 on Chromosorb W HP and detection was carried out by FID. Column temperature was 210 °C, detector temperature was 300 °C, and injection port temperature was 250 °C. Standard solutions in chloroform of major and trace constituents were prepared for determination of retention times and for the quantification of kavalactones. All the peaks were well separated except the peak of yangonin, which was not completely resolved from that of methysticin, and its peak height was not linearly related to sample size (Fig. 2). No interference by polar "tarry" material or non-polar low-boiling compounds was reported and satisfactory reproducibilities were obtained by the proposed method [27].

Smith examined by GC different plant parts (stems, roots, rhizome, leaves) of two cultivars of *P. methysticum* by using 3% OV-101 and 2.5% OV-17 packed glass columns (2 m × 3 mm) [18,28]. Compounds were revealed by a FID detector using a column temperature at 260 °C. Roots, rhizomes, leaves and stems were extracted in ethyl acetate and then analysed. Quantities of kavalactones changed in the different parts

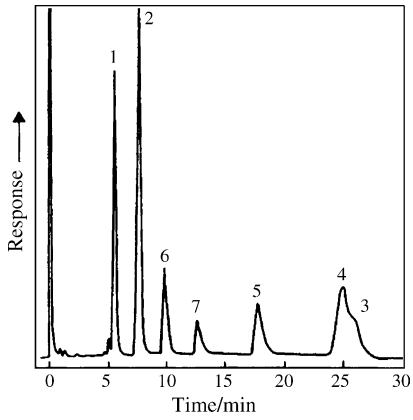


Fig. 2. Gas–liquid chromatogram of a kava-kava extract. Conditions: column, dual 1.5 mm × 4 mm i.d. glass containing 3% m/m of OV-1 on Chromasorb W HP; dual differential flame-ionization detector; N_2 as carrier gas at 300 kPa and 60 ml min^{-1}; H_2 at 150 kPa and 60 ml min^{-1} and air at 180 kPa and 240 ml min^{-1} as fuel gas; column temperature 210 °C; detector temperature 300 °C; injector port temperature 250 °C; integrator sensitivity 0.3–1.0 mV min^{-1}. Peaks: 1 = DIHK, 2 = K, 3 = M, 4 = Y, 5 = DIHM, 6 = DEHK, 7 = THY. Reproduced from [27], with the permission of The Royal Society of Chemistry.

of the plant: kavain and demethoxyyangonin were the major constituents of the hypogeal parts, while dihydrokavain and dihydromethysticine were the major constituents in leaves and stems. No peak for methysticine was identified, even if different GC conditions were tested. However, methysticin which is one of the major components could not be reliably measured by GLC as it decomposes in the injection port [18] and it seemed that liquid chromatography would be a suitable alternative technique.

In the same period, the investigation of different extracts of kava-kava by GC coupled with mass spectrometry analysis was carried out by Duffield and Lidgard [9,29], and Cheng et al. [8]. Electron impact (EI) and methane negative ion chemical ionization (NICI) mass spectrometry were used in order to identify major and minor kavalactones and other lipophilic constituents of the investigated extracts.

Capillary and packed columns were employed in the study to make a comparison of the results. Fused quartz capillary column were BP-1 and BP-10 type; packed columns were filled with OV-17, OV-1 and SP-1000 stationary phase. All the three studies used almost the same conditions. However, capillary column length (8 and 30 m in [9,29], 12 m in [8]), temperature gradient (100–300 °C at 10 °C min^{-1} for BP-1 and BP-17 in [9,29], 100–300 °C at 6 °C min^{-1} for BP-1 and 100–300 °C at 10 °C min^{-1} for BP-17 in [12]), and carrier flow (always helium flow is 1.0 ml min^{-1} except BP-10 helium flow was 0.4 ml min^{-1} in [9]) were slightly different. Normally run time was almost 20 min, except for BP-1 packed column in [8]: in that case the analysis time was almost 33 min. The detector employed was a mass spectrometer using EI ionization and methane NICI. Both detections are useful for the identifications of kavalactones, but NICI gave better results because of greater sensitivity of detection of some selected kavalactones, estimated to be 15 to 20 times that of EI or PICI for the same compounds.

Using these methods, it seems possible to separate many of the known constituents of *P. methysticum*, such as K, DIHK, K, DEHK and DIHM. Although methysticin is one of the major constituents of *P. methysticum*, also in these cases it was never be found at more than trace levels during the course of the analyses, perhaps due to thermal decomposition. It was also possible to detect minor constituents of kava-kava: the structures were resolved by their $[M–H]^+$ ions and fragmentation patterns [8,9,29].

About a decade later, a new paper appeared in the literature concerning GC-MS analysis of an extract obtained by supercritical fluid extraction (SFE) [30]. Analyses were carried out on a capillary PTE-5 fused-silica column. The column temperature program was set as follow: 40 °C for 4 min, linear gradient to 275 °C at 8 °C min^{-1}, constant temperature of 275 °C for 8.5 min, injector temperature was 250 °C. The mass detector was EI and quantification of kavalactones was performed using K as standard. The precision of the method was good in terms of reproducibility of retention time (relative standard deviation of R_t of K was 0.1% nine analyses over a period of a month) and linearity was good in the 10- to 100 μg/ml concentration range [30].

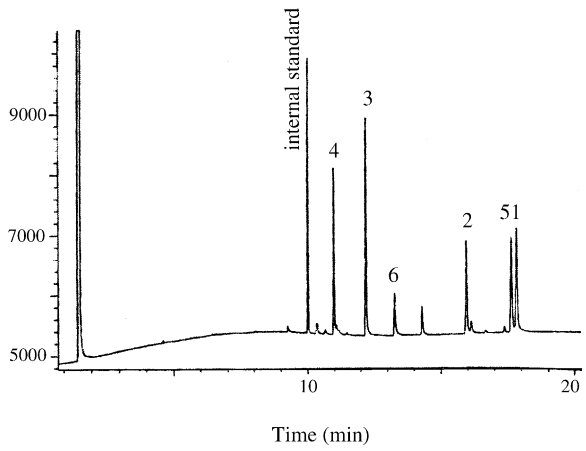


Fig. 3. Gas chromatogram of a kava-kava extract. Conditions: capillary column DB-1 minibore, 0.18 mm i.d., 20 m length, injector (split-splitless): 250 °C, FID detector temperature: 300 °C, temperature gradient: 140 to 230 °C (10 °C min^{-1}), to 280 °C (4 °C min^{-1}) and hold this temperature for 3 min, injected volume: 2 μl, carrier gas (He) flow: 0.5 ml min^{-1}. Peaks: 1 = M, 2 = DIHM, 3 = K, 4 = DIHK, 5 = Y, 6 = DEHK. Reproduced from [31] with the permission of GWV Fachverlage (Vieweg Publishing).

The last paper in the literature concerning GC kava-kava analysis was performed in order to make a comparison of the different analytical methods, i.e. GLC, RP-HPLC and NP-HPLC [31]. For the GC analysis a FID detection was employed using a DB-1 minibore capillary column with linear gradient temperature: 140–230 °C (10 °C min^{-1}), 230–280 °C (4 °C min^{-1}), and 280 °C for 3 min. Peaks were assigned by comparison with standard compounds. Calibration curves were performed for methysticine, dihydromethysticine, kavain, dihydrokavain, dehydrokavain and yangonin each dissolved in acetone and methyl heptadecanoate was used as internal standard. Run time was 24.5 min, but a baseline separation of the major kavalactones was possible in 18 min (Fig. 3) and data obtained were well reproducible [31].

3.2. High-performance liquid chromatography

The first paper concerning HPLC analysis of kava-kava extracts and preparations was published in 1980 by Gracza and Ruff [32]. The analysis was carried out using a Nucleosil 100-5 column eluting with a binary system *n*-hexane–dioxane (85:15). The chosen column and the liquid phase allowed a good separation of kavalactones and their quantitative determination according to the linearity of the calibration whose regression lines run linear. A partial validation of the method was also performed by analysis of the reproducibility of the determination method which was good (i.e. variation coefficient for kavain was ±0.49%).

A few years later an investigation [19] was carried out in order to obtain a full determination of the kavalactones and

to compare normal and RP-HPLC as analytical tools. HPLC separation was carried out both using a variable-wavelength detector at 254 or 355 nm and a fluorimeter having $\lambda_{excitation}$ 337 nm, $\lambda_{emission}$ 460 nm.

For the NP-HPLC analysis an Hypersil column was used eluting with 1.5% acetonitrile in dichloromethane, acetanilide was used as an internal standard. However, the separation of kavain, dihydrokavain and yangonin was insufficient for the quantitative analysis of the crude plant extract. For this reason an alumina Alox T column, using CH_3CN:CH_2Cl_2 (1.5:98.5) as mobile phase, was chosen. Although separation of major kavalactones was improved, yangonin and kavain peaks were still incompletely resolved. Further studies were then carried out using an alumina (Alox T) column with 1% acetonitrile in dichloromethane as solvent. No chromatograms are reported in the article, even if it was pointed out that the order of kavalactone elution changed compared with the silica column as the 7,8-dihydro-derivatives (DIHK and DIHM) were now eluted after the unsaturated lactones (kavain and methysticin). Overall the separation was improved but yangonin and kavain were still incompletely resolved even if they could be discriminated by comparison of the absorptions of the peaks at 254 and 355 nm as only yangonin absorbed at the latter wavelength. Aminoazobenzene was able to be used as an internal standard for both quantitation and identification as it responded at both wavelengths. An alternative selective response for Y and M was able to be obtained by using a fluorimetric detector but the results were not sufficiently reproducible and it was difficult to find a suitable internal standard. Using this separation system it was possible to compare extracts of two cultivars which were virtually identical by GC analysis [18]. The advantage over the previously proposed GLC analysis was that methysticin could be determined by HPLC. In order to try to obtain a completely resolved separation which would remove much of the ambiguity regarding the results on alumina, the kavalactones were examined using an ODS-Hypersil column and elution was performed with methanol–water (55:45). However, the results were disappointing, only small differences in retentions were obtained and the overall separation was poorer than on normal-phase. Finally, focused studies on yangonin revealed that this constituent undergoes to *trans–cis* photoisomerisation both in standard and in extract, so the analysis of kavalactones has to be carried out in absence of light to avoid this reaction, in particular when aqueous or methanolic solutions are employed or the analysis is performed with alumina.

In the same period another paper [33] appeared concerning the normal-phase HPLC analysis was performed using Si 100 column with *n*-hexane–dioxane 18:82 as eluent. The eluent system was very similar to that reported by Gracza and Ruff [32]. Detection was carried out at 250 nm, using 3,4-dimethoxybenzaldeyde as internal standard. The aim of this paper was the quantitative evaluation of several herbal drugs having different provenience (Fidj, Hawaii, Samoa) and some commercial preparations of kava-kava or in combination with other herbal drugs. No data on the validation of the method is reported [33].

A similar analytical method was reported a decade later by Lebot and Levesque [34] for the analysis of several chemiotypes (HPLC analysis conducted on 63 cultivars of *P. methysticum* from the germplasm collection of Vanuatu, Efate Island) using a photodiode-array detector. The column was a Si 60 Superspher and a mixture of hexane–dioxane (4:1) was the mobile phase. The UV detection at 240 nm was used in order to quantify the six major kavalactones representing for 96% of the total lipid extract.

The use of a chiral column, namely, ChiraSpher NT was described by Boonem et al. [35].

This HPLC method was evaluated in order to quantitatively determine kavapyrones in tablets using as eluent 1,4-dioxane–*n*-hexane (18:82, w/w) with an UV detection at 240 nm. A typical chromatogram is reported in Fig. 4 and, although the separation of enantiomers takes 120 min by isocratic elution, using a gradient procedure with shorter retention times is not an alternative. For quantitative determinations using external standards, calibration curves were

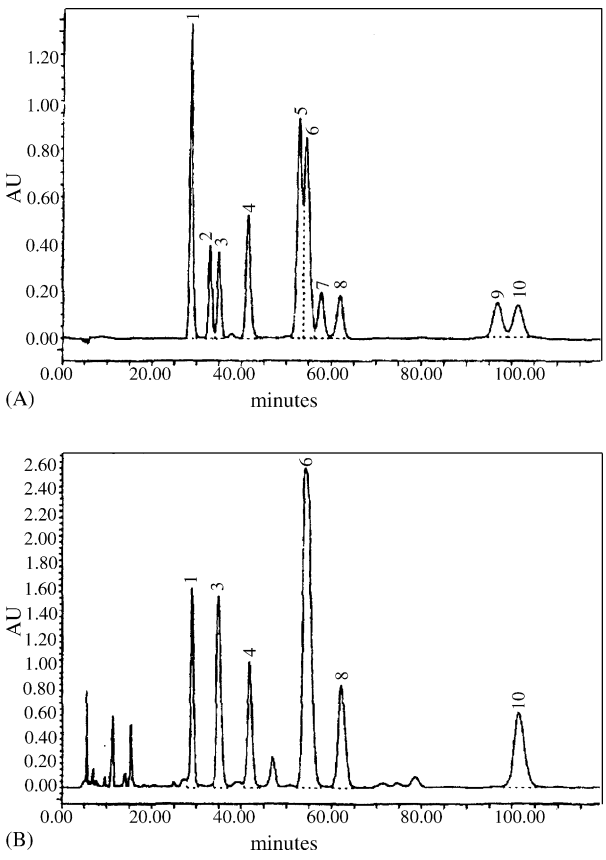


Fig. 4. HPLC chromatogram of synthetic kavalactones (A) and a kava-kava extract (B). Conditions: ChiraSpher NT (4 mm × 250 mm, 5 μm) column; eluent *n*-hexane–1,4-dioxane (82:18) at a flow rate of 0.5 ml min^{-1} for 120 min; UV detection at 240 nm, temperature 15 °C. Peaks: 1 = DEHK, 2 = (−)-DIHK, 3 = (+)-DIHK, 4 = Y, 5 = (−)-K, 6 = (+)-K, 7 = (−)-DIHM, 8 = (+)-DIHM, 9 = (−)-M, 10 = (+)-M. Reproduced from [35], with the permission of Elsevier.

established for every genuine kavalactone. The accuracy of the quantitative kavalactone determination was verified by the analyses of six samples ranging from 80 to 120% of the analysed value of 39.6 mg/tablet. The mean of recovery yielded a value of 99.7% with a confidence interval (P = 95) of $\mu = 99.7 \pm 1.78\%$. The R.S.D. value was 1.71%.

Further studies have been carried out also using reversed-phase separation technique, in some cases by development of gradient elution systems: in this way it was possible to perform good separation of kavalactone constituents in a shorter time than the isocratic elution system.

He et al. investigations [7] used an ODS-Prodigy column and the mobile phase was: solvent A = H_2O (0.25% HOAc), solvent B = MeOH. A linear gradient elution was applied: 20–50% B at 0 to 8 min, 50–70% B at 8 to 25 min, 70–100% of B at 25 to 40 min, 100–20% of B at 40 to 45 min. The HPLC instrument was coupled with a MS (EI) and a photodiode-array detector. Seven major kavalactones were unambiguously identified based on the R_t and MS data, compared with standard compounds. Another six, minor peaks were identified, based on the comparison of R_t, MS data with isolated compounds. The use of a RP-HPLC in this work was justified because electrospray usually requires polar solvents and ions of analyte can be generated in solution before nebulization so as to increase sensitivity of the mass spectrometer. For this reason, adding acetic acid to water did not affect the separation of HPLC, but increased the sensitivity of the ES-MS due to the better ionization of kavalactones.

Another paper concerning the use of RP-HPLC but using isocratic conditions is by Shao et al. [36] who performed the analyses on YMCbasic column, with an isocratic mobile phase of MeOH:CH_3CN:H_2O:CH_3COOH (20:20:60:0.1, v/v/v/v) coupled with a mass spectrometer with an APCI source. The average recoveries were good (between 98.2 and 100.6%), the detection limit was between 0.5 and 1.1 μg/ml, and precision tests showed average relative standard deviations less than 3.5%, suggesting that the method has excellent precision. The method used an isocratic mobile phase that

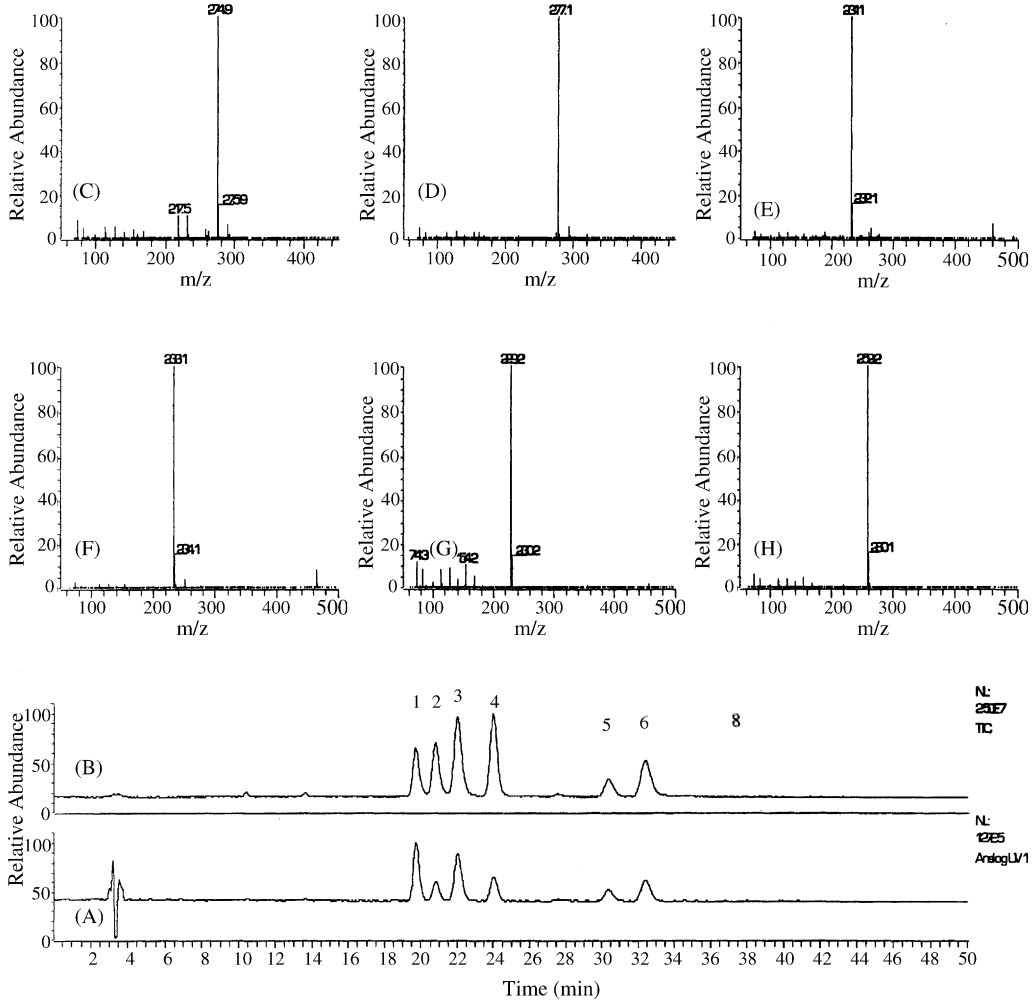


Fig. 5. HPLC-UV-MS chromatogram of a kava-kava extract. Conditions: column YMCbasic RP, 25 cm × 4.6 mm i.d., 5 μm particle size; isocratic mobile phase MeOH–CH_3CN–H_2O–CH_3COOH (20:20:60:0.1, v/v) at a flow rate of 1 ml min^{-1}, column temperature 40 °C; detection 220 nm. (A) UV trace, (B) total ion trace, (C) MS spectrum of peak 1 (M), (D) MS spectrum of peak 2 (DIHM), (E) MS spectrum of peak 3 (K), (F) MS spectrum of peak 4 (DIHK), (G) MS spectrum of peak 5 (DEHK), and (H) MS spectrum of peak 6 (Y). Reproduced from [36], with the permission of Elsevier.

was very favourable 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-kava samples. The HPLC method described in this paper by Shao and coworkers provides baseline separation of all six major kavalactones (Fig. 5) and permitted the quantification of the six kavalactones rapidly and accurately both in plant extracts and in preparations using a single run [36].

Ganzera and Khan [31] carried out the separation of kavalactones by GLC and HPLC (both normal- and reversed-phase, Fig. 6) on kava-kava extracts. In this study the normal-phase separation was performed on a Luna SI column with isocratic *n*-hexane–1,4-dioxane (85:15) and the resolution of dihydromethysticin and kavain were improved, compared to Hänsel system [33]. Reversed-phase separations were carried out on a Luna C_8 column. Mobile phase was an isocratic mixture of H_2O:CH_3CN:reagent alcohol (65:20:15). Reagent alcohol was a mixture of ethanol, methanol and isopropanol (90.6:4.5:4.9). Run time was 30 min for normal-phase separation and 40 min for reversed-phase. If reagent alcohol is exchanged with methanol, peaks 5 and 6 are not resolved at room temperature; with only water/acetonitrile as mobile phases 1 and 2 are merged. Pure ethanol instead of reagent alcohol results in a poorer resolution of 1–2 and 5–6. Kavalactones could be detected at very low concentrations, considering that only 5 μl of sample were injected. All compounds were easily detectable from concentration of 4 μg/ml upwards; kavain, with the highest UV absorption at 246 nm, was even detectable at concentrations of 0.5 μg/ml.

As a conclusion the authors pointed out that all the three systems were suitable for the separation and quantification of the main kavalactones. Very consistent analytical results were obtained, but each of the methods revealed advantages and disadvantages. A separation by reversed-phase method takes nearly 40 min, but the obtained results are highly reproducible (1.6% maximum standard deviation); no internal standard is needed and the detection limit is very low for all compounds (4 μg/ml). Not considering the differences in column length and particle size (and consumption of the mobile phase) the separation by normal-phase is slightly better. All peaks are well resolved, analysis time is 10 min shorter, but the method is less sensitive. As the standard deviation is generally higher (3.5%), the use of an internal standard is advised. To avoid most of the disadvantages for the normal-phase method the use of a shorter column and 3 μm material should be helpful. Finally, a separation by GC only needs 18 min, and by including an internal standard, the obtained results are well reproducible. The main disadvantage of this method is its rather low sensitivity [31].

HPLC analysis of the six major kavalactones was also carried out on an analytical Spherisorb ODS column [37]. The samples were eluted with 22% acetonitrile, 18% methanol

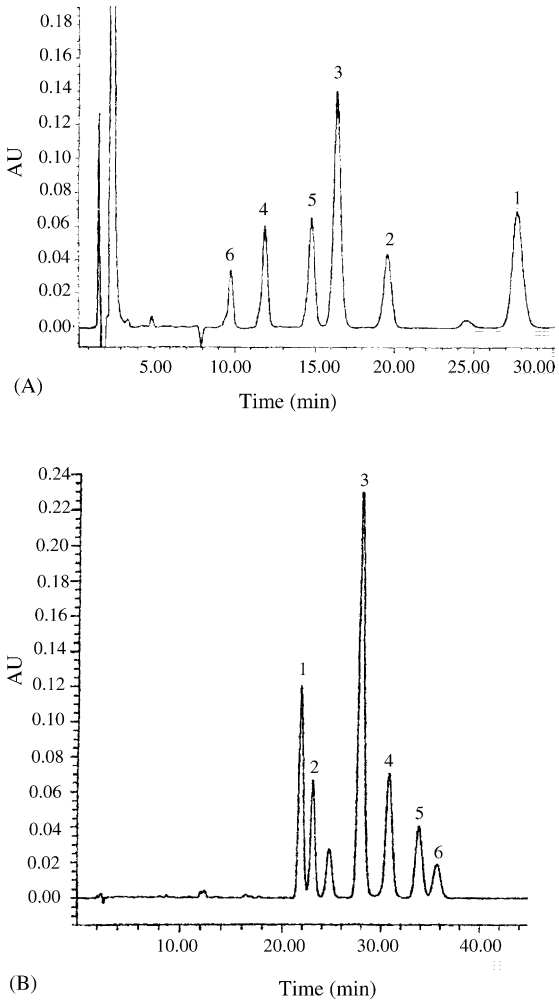


Fig. 6. NP-HPLC-UV (A) and RP-HPLC-UV (B) chromatograms of a kava-kava extract. Conditions for A: column Luna SI, 5 mm particle size, 150 mm × 4.6 mm; mobile phase *n*-hexane–1,4-dioxane (85:15) at a flow rate of 1.5 ml min^{-1} for 35 min, detection at 246 nm, injection volume 10 μl, ambient temperature. Assignment of peaks: 1 = M, 2 = DIHM, 3 = K, 4 = DIHK, 5 = Y, 6 = DEHK. Conditions for B: column Luna C_8, 3 mm particle size, 100 mm × 4.6 mm; mobile phase: isocratic mixture of H_2O:CH_3CN:reagent alcohol (65:20:15) at a flow rate of 0.5 ml min^{-1} for 45 min, detection at 246 nm, injection volume 5 μl, ambient temperature. Reproduced from [31], with the permission of GWV Fachverlage (Vieweg Publishing).

and 60% H_3PO_4 (50 mM) within 50 min. Y and DEHK were detected at a wavelength of 360 nm, whereas the other four kavalactones were measured at 240 nm. Each sample was separately extracted at least twice and analysed by HPLC. Analytical determinations are given as mean ± standard deviation.

Important studies on the optimisation of the analysis of kavalactones were carried out by Schmidt and Molnar [38]. Using a computer simulation and starting from four experiments, the software allowed optimisation of gradient time t_G and T temperature. Changing variables such as type of organic modifier, the eluent pH and the flow rate, the optimisation resulted in resolution $R_s > 1.5$ for all kavalactones and the two additional new bands. 2-Propanol was shown to play

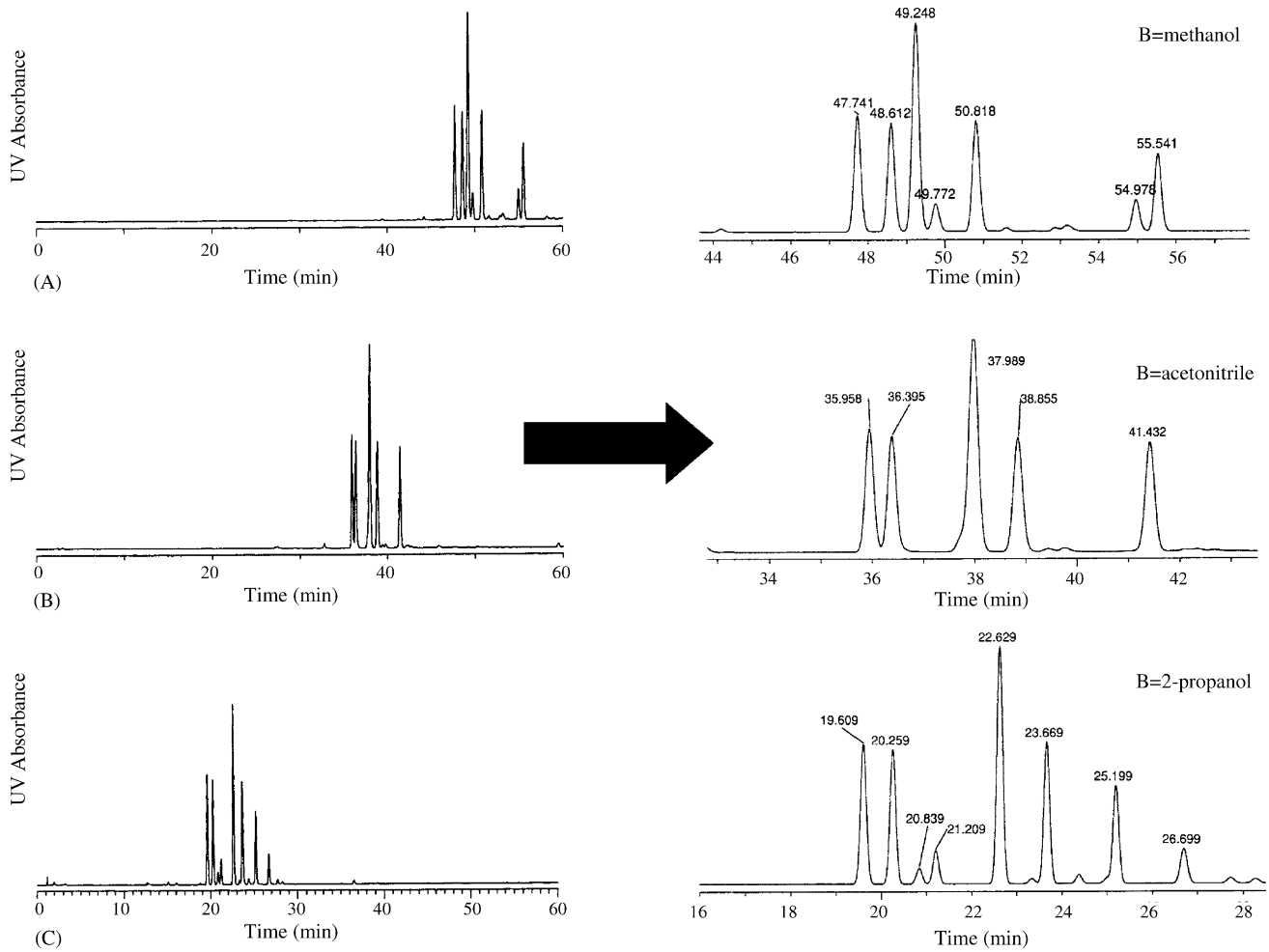


Fig. 7. HPLC-UV chromatograms and relevant parts performed with t_G/T values of 90 min/60 °C using MeOH (A), CH_3CN (B) and 2-propanol (C) as the organic modifiers. Conditions: column Luna C_{18} 250 mm × 4.6 mm, 5 mm particle size; mobile phase: unbuffered (pH 6.15) water (A), organic modifier (B) with a linear gradient from 10 to 90% B. DH: dihydro; DM: desmethoxy; U: unknown. Reproduced from [38], with the permission of Elsevier.

an important role as organic modifier: the retention time of kavalactones is the shortest and the separation is the best possible, acetonitrile and methanol deliver hidden components and long analysis times. In methanol, two bands cannot be separated at all. Separation of kavalactones was performed on a Luna C_{18} column using two different linear gradients of mobile phase: 10–90% of solvent B in 30 or in 90 min. Solvent A was always H_2O unbuffered, solvent B was: CH_3CN, MeOH and 2-propanol. Optimisation methods involved the selection of experimental conditions for adequate separation an acceptable retention time (Fig. 7). Finding the overall optimum conditions is therefore often a compromise between contradictory objectives [38].

Recently, HPLC analysis has been used to investigate the variability of chemical composition of the herbal drug according to geographical origin [39]. In this study NP-HPLC analysis using a Nucleosil 50 column was employed with an isocratic mobile phase of hexane:1,4-dioxane:methanol (85:13:2). Quantification was carried out at a single wavelength of 245 nm. The experimental error was estimated to be approximately ±0.5%. This is the first time that in normal-

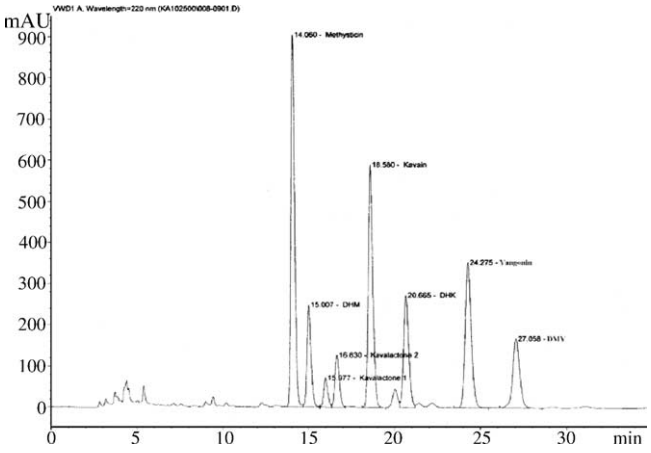


Fig. 8. HPLC chromatogram of a kava-kava extract. Conditions: YMCbasic column, 4.6 mm × 250 mm, 5 μm, column temperature: 40 °C, flow rate: 0.6 ml min^{-1}, mobile phase: isocratic 0.1% phosphoric acid:isopropyl alcohol:acetonitrile (64:16:20, v/v). Detection: 220 nm (246 nm alternatively to improve selectivity), injection volume: 5 μL, run time: 30 min. Reproduced from INA method 101.006, Institute for Nutraceutical Advancement, 2002, http://www.nsfina.org/methods/kavaset.html.

Table 1
Experimental conditions for the optimisation of kavalactone separation by supercritical fluid chromatography

Column	Spherisorb NH_2	C_4 protein	Diphenyl	Altima CN	Supelcosil LC-diol
Column temperature	60 °C	80 °C	80 °C	60 °C	60 °C
Flow rate	2.0 ml min^{-1}	2.5 ml min^{-1}	2.0 ml min^{-1}	2.0 ml min^{-1}	2.0 ml min^{-1}
Mobile phase	A = CO_2 B = MeOH	A = CO_2 B = MeOH (1% isopropylamide)	A = CO_2 B = MeOH	A = CO_2 B = MeOH	A = CO_2 B = MeOH
Gradient	A 98% (0–3 min) to A 90% (0.4% min^{-1} step)	A 98% (0–3 min) to A 90% (0.4% min^{-1} step)	A 98% to A 93/7% (0.1% min^{-1} step)	A 98% (0–3 min) to A 90% (0.4% min^{-1} step)	A 98% (0–3 min) to A 90/10% (0.4% min^{-1} step)
Pressure (atm)	125	125	125 (0–3 min); 125–195 (5 atm min^{-1})	125	125

phase separation organic modifier is reported, however no chromatograms are included in the paper to evaluate the separation of the kavalactones [39].

The last report on HPLC analysis of kavalactones was designed to demonstrate the potential application of PFE in the extraction of the kavalactones from *P. methysticum*. HPLC-UV and HPLC-MS were used to analyze kavalactones, to determine the selectivity and efficiency of extraction parameters, and to verify the integrity of the chemical constituents throughout the extraction process [40]. For

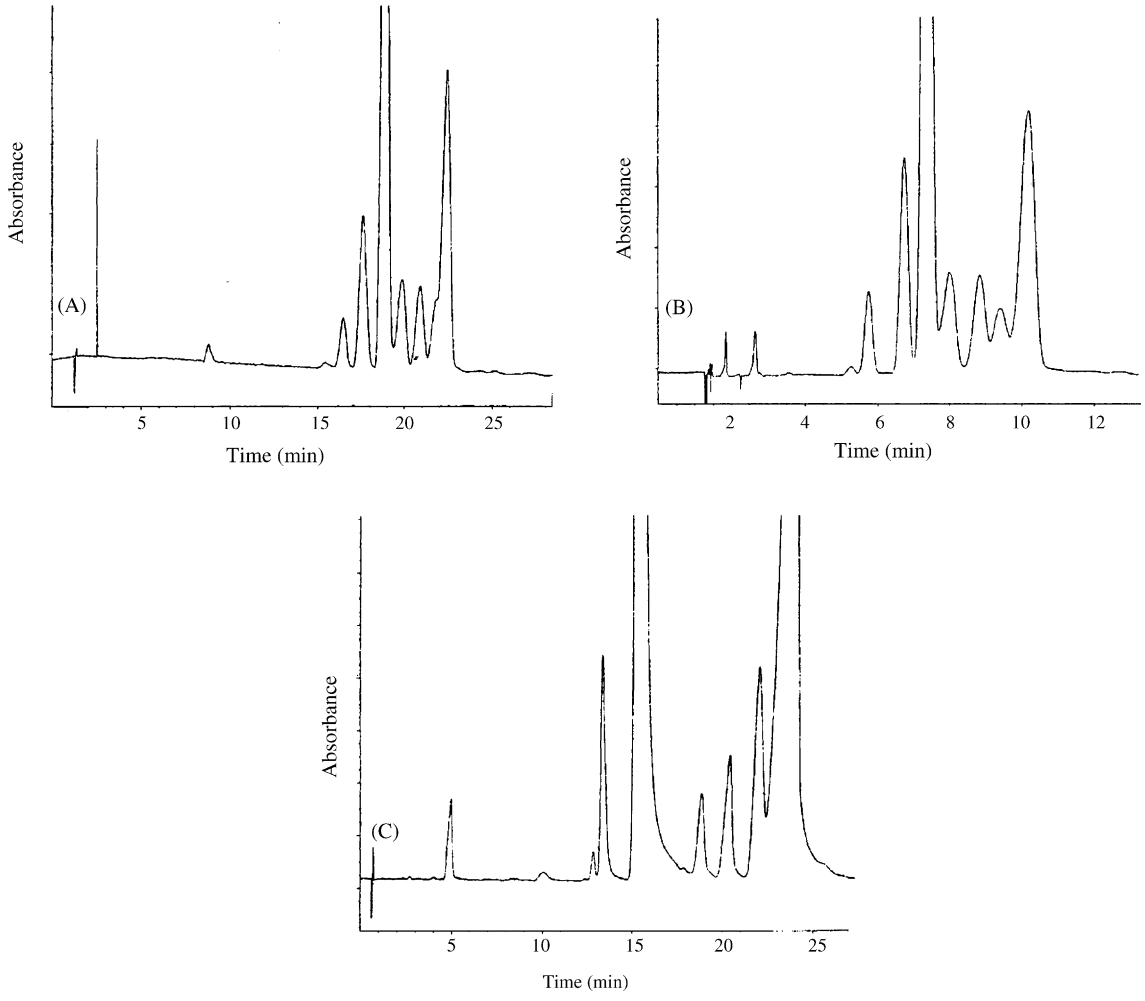


Fig. 9. SFC chromatograms of a kava-kava extract. Conditions for A: NH_2 column, 250 mm × 4.6 mm, 5 μm d_p; pressure 125 atm, 60°C, 2 ml min^{-1}; modifier program: 98/2 CO_2/MeOH for 3 min and then increased to 90/10 CO_2/MeOH at rate of 0.4% min^{-1}. Conditions for B: protein C_4 column, 250 mm × 4.6 mm, 5 μm d_p; pressure 125 atm, 60 °C, 2.5 ml min^{-1}; modifier program: 98/2 CO_2/MeOH containing isopropylamine for 3 min and then increased to 90/10 CO_2/MeOH at rate of 0.4% min^{-1}. Conditions for C: diphenyl column, 250 mm × 4.6 mm, 5 μm d_p; pressure: 125 atm for 3 min and then increased to 195 atm at rate of 5 atm min^{-1}, 80 °C, 2 ml min^{-1}; modifier program: 98/2 CO_2/MeOH for 3 min and then increased to 93/17 CO_2/MeOH at rate of 0.1% min^{-1}. Reproduced from [42], with the permission of GWV Fachverlage (Vieweg Publishing).

the HPLC analysis the validated INA method [41] was used. A YMC J'sphere ODS-H80 basic reverse phase column was employed, eluting isocratically with a mixture of 0.1% H_3PO_4 (60.9%), CH_3CN (20.6%) and isopropyl alcohol (18.5%). The six major kavalactones were well resolved in the chromatogram as reported in the Fig. 8 [41].

3.3. Supercritical fluid chromatography

Supercritical fluid chromatography (SFC) is an useful separation process that allows extraction and separation of natural constituents of herbs without employing large quantities of solvents, which may also be toxic, and without thermal decomposition. The most common supercritical solvent is CO_2: it is not toxic and it is also easy to remove. An efficient analytical separation with supercritical fluid was considered to be advantageous in preparation for future scale-up work to isolate large quantities of each kavalactone. For this purpose, Ashraf-Khorassani et al. [42] studied how different stationary phases, pressures, temperatures and modifier concentrations influence the separation of kavalactone constituents, in order to optimize it. In their study five different columns were tested, with different conditions of flow rate, column temperature of column and mobile phase gradient. In four experiments pressure was maintained constant; in one linear gradient was employed. Detection of kavalactones was monitored with a UV detector set at 254 nm. Details concerning the experimental conditions are reported in Table 1.

Separation of seven kavalactones from kava-kava root extract was performed using methanol as modifier of the supercritical CO_2. Optimisation of kavalactone separation was achieved using amino and protein C_4 columns which almost provided baseline separation of all kavalactones. Peaks resolution was performed better by C_4 columns compared to NH_2 and diphenyl (Fig. 9). Feasibility of SFC for the separation of kavalactones was demonstrated but no data concerning validation of the methods is reported [42].

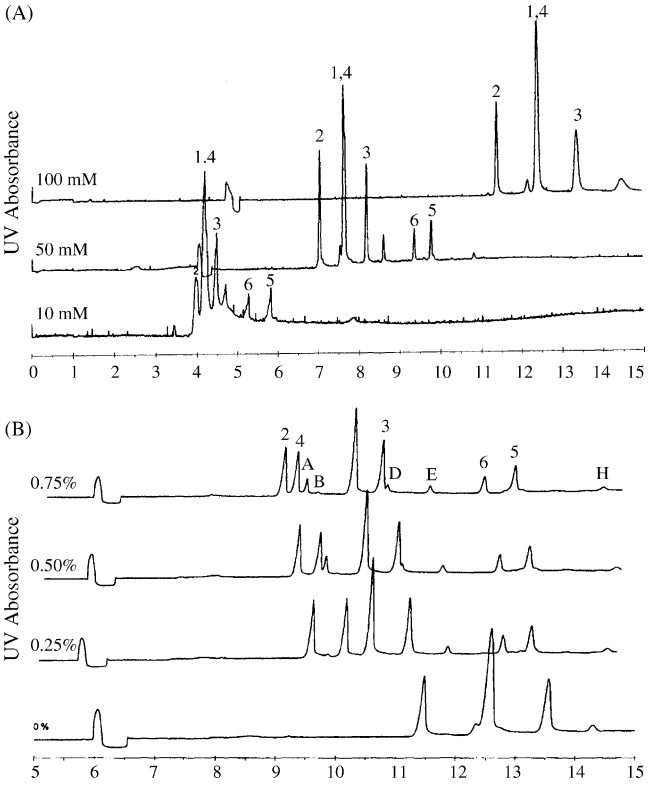


Fig. 10. MECK separations of kavalactones from a kava-kava extract. (A) Influence of TDCh concentration [100 mM borate buffer, pH 8.3, 30 kV, capillary: 77 cm (70 cm to detector) × 50 μm i.d., 240 nm] on the R_t of kavalactones. Assignment: 1 = K, 2 = DIHK, 3 = M, 4 = DIHM, 5 = Y, 6 = DEHK. (B) Influence of β-CD concentration [100 mM borate buffer, pH 8.3, 50 mM TDCh, 30 kV, capillary: 77 cm (70 cm to detector) × 50 μm i.d., 240 nm] on the R_t of kavalactones. Assignment: 1 = K, 2 = DIHK, 3 = M, 4 = DIHM, 5 = Y, 6 = DEHK, A, B, D, E, H = unidentified peaks. Reproduced from [43], with the permission of Elsevier.

4. Electromigration methods

The only paper concerning the use of electromigration methods is by Lechtenberg et al. [43] who developed a micellar electrokinetic chromatography (MEKC) method for the qualitative and quantitative determination of the active principles of *P. methysticum* present both in dry herbal extracts and drug preparations. To separate the major constituent of dry extracts of kava-kava plant, fused-silica capillaries with a borate buffer containing sodium-taurodeoxycholic acid and β-cyclodextrin were used; 4-hydroxybenzoic acid methyl ester was the internal standard. Calibration was carried out for each enantiomeric major kavalactone, adding the internal standard. Diode-array detection of kavalactones was set at 254 nm (for quantification) and 350 nm; voltage was at 30 kV; temperature was 27 °C. Composition of the running buffer was: 50 mM of sodium taurodeoxycholate (TDCh) in 100 mM bo-

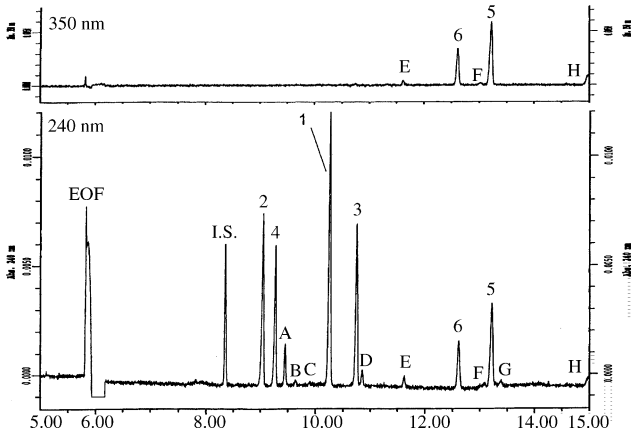


Fig. 11. MEKC separation of kavalactones from a kava-kava extract. Conditions: 100 mM borate buffer, pH 8.3, 50 mM TDCh, as eluent; applied voltage 30 kV; capillary: 77 cm (70 cm to detector) × 50 μm i.d.; detection at 240 and 350 nm. Assignment: 1 = K, 2 = DIHK, 3 = M, 4 = DIHM, 5 = Y, 6 = DEHK, A–H = unidentified compounds. Reproduced from [43], with the permission of Elsevier.

rate buffer (100 mM H_3BO_3:100 mM $Na_2B_4O_7$, 9:1; pH 8.3) containing 0.75% of (m/v) of β-cyclodextrin. The final pH of the running buffer was 7.9. The limit of quantification for kavain was 0.05 mg/ml; for the other constituents it was 0.1 mg/ml. The limit of detection was 0.01 mg/ml. Good separation and quantification were available in 15 min. The concentration of the TDCh acid and β-cyclodextrin influenced the migration time and the resolution of the peak of both major and minor constituents of the herbal extract (Fig. 10). The developed method is rapid and selective, it allowed the separation and determination of the kavalactones in crude extract and drug preparation (Fig. 11). It is robust, sensitive enough for standard method and the recovery is good [43].

5. Conclusions

The determination of kavalactones has been performed by GC and normal and reversed-phase HPLC. Very consistent analytical results have been obtained, but all of these methods have some limitations concerning separation time or peak resolution.

GC analysis has been used both for qualitative and quantitative purposes using, first, packed columns and then capillary columns. FID has generally been used as detector, but gas chromatography with electron impact and methane chemical ionization mass spectrometry has been very useful in the identification of minor constituents and also to identify some of the human urinary metabolites of kavalactones. All the studies have pointed out the possibility of using GC for the separation and quantification of major constituents, even if methysticin and yangonin, which are two of the major components, are not able to be separated. In addition, the high temperature of the injection port causes the decomposition of methysticin itself.

Concerning HPLC analyses, normal-phases such as Sigel and alumina were tested first. However, in addition to some advantages over reverse-phase methods, such as a shorter time of analysis and a slightly better separation of constituents, NP-HPLC is less sensitive and the use an internal standard due is advisable to the higher standard deviations. It also has a few difficulties related the use of undesirable toxic and volatile organic solvents, and the need for long equilibration times. Furthermore, sample preparation for normal-phase column chromatography is generally complicated by the fact that the injection sample must be free of all traces of water.

Therefore, the need for a reversed-phase HPLC method which is favoured in modern analytical methods has become evident. The oily properties of kava-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 have been carried out.

RP-HPLC analyses are highly reproducible, no internal standard is needed and the detection limit is very low for all compounds. It has been demonstrated that 2-propanol is the best eluent for the separation of the kavalactones. Acetonitrile and methanol deliver hidden components and long analysis times. Further reduction of analysis time is possible by adapting a linear gradient and increasing the flow-rate in order to obtain the separation in about 15 min for eight major components. The use of chiral phases gives very impressive separation but it is too time-consuming for routine analysis.

However, quantitative analysis of the kavalactones by liquid chromatography needs to be carried out in the absence of light to prevent the isomerisation of yangonin in standards or extracts, particularly if aqueous or methanolic solutions are used or the analysis is performed with alumina.

Concerning the HPLC detectors, primary detection of kavalactones can be performed by UV, and secondary detection by mass spectrometry or fluorescence detector. UV at wavelengths between 240 and 254 nm is acceptable for the detection of all kavalactones, while the wavelength at 355–360 nm is only useful for detecting yangonin. MS is also very useful because it gives information about constituent structures, especially in the search for new derivatives.

Two other methods have been proposed for the analysis of kavalactones: supercritical fluid chromatography and micellar electrokinetic chromatography. They can both represent selective and rapid analytical methods for the six major kavalactones, however these approaches are too preliminary to be proposed as alternative methods.

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