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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF KAVA LACTONES FROM *PIPER METHYSTICUM*

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SUMMARY

The kava lactones from different plant parts of *Piper methysticum* have been examined using normal and reversed-phase high-performance liquid chromatography. An unexpected photoisomerisation of yangonin in the mobile phase occurred during sample preparation which complicated the analysis.

INTRODUCTION

The tropical shrub *Piper methysticum* Forst (Piperaceae) is widely cultivated in the South Pacific where it is known as 'awa, kawa, kava, and yanqona' and is important as a folk medicine and as the basis of a ceremonial and social drink. The roots and stems have attracted considerable attention because they contain a series of α-pyrones (kava lactones I–VI), which have pharmacological activity with anticonvulsive, antiepileptic, fungistatic, and local anaesthetic effects[1–5].

As well as the kava lactones, a number of characteristic alkaloids, and flavanoids (flavokawains) have been reported. Although the individual lactones have been widely studied little work has been carried out on the concentration and distribution of these constituents in the plants. A number of methods have been developed for the determination of the major constituents including methysticin (VI), yangonin (IV), and kawain (II), in purified extracts or synthetic mixtures using thin-layer chromatography (TLC) or spectroscopic methods[6–9]. Gas–liquid chromatography (GLC) was first used in 1971 as a qualitative method and lead to the identification of additional constituents in the roots[10] but instrumental methods have only recently been applied to quantitative analysis with a GLC study of the proportions of kava lactones and alkaloids in different plant parts[11,12] and of lactones in the rhizomes[13]. However, methysticin which is one of the major components cannot be reliably measured by GLC as it decomposes in the injection port[11,13] and it seemed that liquid chromatography would be a suitable alternative technique.

The present paper reports studies to try to obtain a full determination of the lactones by using both normal and reversed-phase high-performance liquid chro-

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matography (HPLC). The work was hampered by a previously unreported photoisomerisation of yangonin which interfered with the assays. While this work was in progress an alternative HPLC method using a silica gel column was reported as part of a pharmacological study[14].

EXPERIMENTAL

Plant material

Roots, stems, and leaves of *Piper methysticum* were collected in Suva, Fiji. The dried powdered plant material was extracted with ethyl acetate in a Soxhlet extractor and the extract was passed through a short silica column before analysis.

Standards

Authentic samples of the kava lactones were obtained from the plant extracts by TLC on silica gel and alumina and were identified by a comparison of their UV and IR spectra and melting points with reported values[15].

Synthetic yangonin for the photoisomerisation studies was prepared from anisaldehyde and 4-methoxy-6-methyl-2*H*-pyran-2-one[16].

High-performance liquid chromatography

HPLC separations were carried out using a Pye Unicam XPS pump, Cecil Instruments 2012 variable-wavelength detector at 254 or 355 nm and a Perkin-Elmer 1000 fluorimeter with HPLC detector cell, $\lambda_{excitation}$ 337 nm, $\lambda_{emission}$ 460 nm. Samples were injected using a Rheodyne 7125 valve on to Shandon Southern columns, which had been slurry packed with Alox T 10 μm (25 cm × 5 mm I.D.) (Merck, Darmstadt, F.R.G.), Hypersil 5 μm (10 cm × 5 mm I.D.) and ODS-Hypersil 5 μm (10 cm × 5 mm I.D.) (Shandon Southern, Runcorn, U.K.). Solvents were HPLC grade (Fisons, Loughborough, U.K.).

RESULTS AND DISCUSSION

In order to compare the full profile of kava lactones in different plant parts and cultivars of *P. methysticum* from Fiji, HPLC was examined as this would avoid the problem of methysticin decomposition found in GLC[11].

Normal-phase separation

Because much of the reported work on the isolation of the kava lactones had used TLC on silica gel or alumina, initial HPLC studies were carried out using normal-phase systems. On a Hypersil column the major lactones were eluted using 1.5% acetonitrile in dichloromethane, with acetanilide as an internal standard (Table I). However, the separation of kawain, dihydrokawain and yangonin was insufficient for the quantitative analysis of the crude plant extracts. Further studies were then carried out using an alumina (Alox T) column with 1% acetonitrile in dichloromethane as solvent (Table II). The order of elution of the lactones changed compared with the silica column as the 7,8-dihydro-derivatives (III and VII) were now eluted after the unsaturated lactones (II and VI). Overall the separation was improved but yangonin and kawain were still incompletely resolved. However they could be dis-

TABLE I

LIQUID CHROMATOGRAPHIC SEPARATION OF KAVA LACTONES ON A HYPERSIl COLUMN

Eluent, acetonitrile–dichloromethane (1.5:98.5).

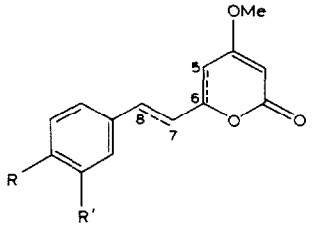
Compound	*k'*
Kawain (II)	9.2
Dihydrokawain (III)	10.0
Yangonin (IV)	11.2
Methysticin (VI)	16.0
Dihydromethysticin (VII)	16.8
Acetanilide (internal standard)	17.9

TABLE II

SEPARATION OF KAVA LACTONES ON ALOX T COLUMN

Eluent, acetonitrile–dichloromethane (1:99).

Compound	*k'*	*Composition of extracts (%)*		
		Root	*Stem*	*Leaf*
Desmethoxyyangonin (I)	1.80	21.6	1.8	3.0
Dihydrokawain (III)	2.40	17.1	23.2	69.8
Yangonin (IV)	2.93	0.8	0.8	1.2
Kawain (II)	3.13	34.5	0.6	2.5
Dihydromethysticin (VII)	4.80	5.3	59.6	22.5
Methysticin (VI)	7.13	20.8	13.9	0.8
Aminoazobenzene (internal standard)	1.20			



		R	R'	5–6	7–8	
I	Desmethoxyyangonin	–H	–H	C=C	C=C	
II	Kawain	–H	–H	C—C	C=C	
III	Dihydrokawain	–H	–H	C—C	C—C	
IV	Yangonin	–OMe	–H	C=C	C=C	*trans*
V	*cis*-Yangonin	–OMe	–H	C=C	C=C	*cis*
VI	Methysticin	$-OCH_2O-$		C—C	C=C	
VII	Dihydromethysticin	$-OCH_2O-$		C—C	C—C	

criminated by comparison of the absorptions of the peaks at 254 and 355 nm as only yangonin absorbed at the latter wavelength. Aminoazobenzene could be used as an internal standard for both quantitation and identification as it responded at both wavelengths. An alternative selective response for yangonin and methysticin could be obtained by using a fluorimetric detector but the results were not sufficiently reproducible and it was difficult to find a suitable internal standard. The other major components of the plant, the flavokawains, were also injected on to the alumina column but were retained, and as the alkaloid pipermethystine had been found to decompose on alumina it was not studied[11].

Using this separation system it was possible to compare the different plant parts. As in the GLC study the two cultivars were virtually identical[11] and only one set of results is given (Table II). A comparison of the composition of the plant parts with the results of the GLC study showed that generally the same components were important in each case but with the advantage that methysticin could now be determined. However, the proportion of yangonin in the lactones detected by HPLC was much smaller than expected. In the GLC study over 16% was found in the leaves and 12% in the stems, the TLC study found *ca.* 9% on the stems[6] and the HPLC study on silica found 7% in the rhizomes[14]. Although the samples of standard yangonin gave only one peak at 254 and 355 nm, when the extract was examined two peaks were found at the latter wavelength. As well as the yangonin peak at $k' = 2.96$, a second much larger peak was present at $k' = 2.00$, which did not correspond to any of the other lactones. At 254 nm this peak would be unresolved from the desmethoxyyangonin peak at $k' = 1.80$. These results raised doubts about the applicability of this method and prompted an examination of separations on a reversed-phase system. From this subsequent work, which is described below, it is possible that the extra peak on alumina is *cis*-yangonin formed in the extract on standing but which is absent from the standard because that was prepared from a crystalline sample. This would cause the proportion of yangonin to be underestimated as its concentration is based on the 355-nm peak and hence the concentration of the kawain determined by difference would be overestimated. In the recently reported analysis using hexane–dioxan (85:15) and a silica column these lactones were fully resolved and no problems with multiple peaks were noted[14].

Reversed-phase separation

In order to try to obtain a completely resolved separation which would remove much of the ambiguity regarding the results on alumina, the kava lactones were examined using an ODS-Hypersil column and elution with methanol–water (55:45) (Table III). However the results were disappointing, only small differences in retentions being obtained and the overall separation was poorer than on normal phase. When the plant extracts were studied it was found that an extra major peak was present that did not correspond to any of the lactones or other plant constituents.

Although all the standard lactone solutions prepared in the mobile phase gave one peak when they were first examined, if the yangonin solution was re-examined after it had been standing for some time two peaks were present. The ratio of the peaks changed on standing and the presence of an impurity or decomposition product was suspected, although there are no reports of yangonin being unstable. Repeated attempts to purify yangonin by TLC failed to remove this peak. As it was felt that

TABLE III

SEPARATION OF KAVA LACTONES ON ODS-HYPERSIL COLUMN

Eluent, methanol–water (55:45).

Compound	*k'*
Methysticin (VI)	8.8
Dihydromethysticin (VII)	9.1
Kawain (II)	9.2
Dihydrokawain (III)	10.3
Desmethoxyyangonin (I)	15.0
Yangonin (IV)	16.3
cis-Yangonin (V)	11.4

it could possibly be a closely related natural impurity present in *P. methysticum*, yangonin was synthesised by a published method[16]. The NMR and IR spectra and melting point agreed with the literature values[15,17] and confirmed that the sample was homogeneous. When a sample was prepared in the mobile phase and examined by HPLC two peaks at $k' = 11.4$ and 16.3 were obtained, corresponding to the peaks in the natural yangonin sample and extract. If the solution of synthetic yangonin was examined immediately after the crystals had been dissolved only the peak at $k' = 16.3$ was obtained but within a short time two peaks were again detectable and again an equilibrium mixture was obtained. The new peak had a similar response at 254 and 355 nm to yangonin but a much weaker fluorescence response. The speed and equilibrium of the reaction was unaffected by heat or pH but differed with the solvent. The change occurred rapidly in aqueous methanol solutions but in organic solvents such as chloroform virtually no change occurred.

Eventually it was discovered that the reaction was photochemically induced as samples prepared, stored and injected in the dark gave only one peak at $k' = 16.3$. The solutions were stable for prolonged periods in the dark but within minutes in daylight two compounds could be detected. Attempts to isolate the individual compounds were hampered because on working up the fractions each compound apparently re-equilibrated to give the mixture. Storing the mixture in the dark did not cause the reformation of the original compound, although on crystallisation from an organic solvent only yangonin appeared to be present.

As it has been reported that the desmethoxyyangonin (I) isolated from *Aniba* species undergoes a *trans* to *cis* photochemical isomerisation[18] it was thought that a similar reaction could be occurring in the present study. Attempts to obtain pure *cis*-yangonin (V) by crystallisation failed as only the *trans* form was deposited. By rapidly evaporating a mixture of isomers in methanol so that crystallisation did not have time to occur, a sample could be prepared in deuterochloroform for NMR spectroscopy. As well as the expected *trans*-olefinic protons at 7.17 and 6.20 ppm ($J = 15$ Hz), there were signals at 6.58 and 5.72 ppm ($J = 10$ Hz) corresponding to a *cis*-double bond. These differences were similar to those in the stillbenes (*trans* 7.10 ppm and *cis* 6.55 ppm)[19]. However the differences were smaller than those found for desmethoxyyangonin (*trans* 7.45 and 6.56 ppm $J = 15$ Hz and *cis* 4.40 and 4.33 ppm $J = 2.2$ Hz) in which steric hindrance has apparently eliminated the double-bond character of the 7,8-bond[18].

HPLC was used to monitor the progress of an equilibration reaction of yangonin. After 15 min in daylight a solution prepared from the crystalline *trans*-yangonin contained a mixture with a *trans–cis* ratio of 62:38 and equilibrium was reached in *ca.* 90 min with a final ratio of 39:61, based on the area of the *trans* peak. This problem of photoisomerisation is presumably particularly noted with yangonin as it is the only kava lactone to absorb in the visible region (λ_{max} 357 nm). Presumably the problem has not been observed in previous studies even after prolonged standing as most work has primarily used organic solvents in which the isomerisation is very slow and usually samples were prepared from crystalline material, which seems to be exclusively the *trans* form.

CONCLUSIONS

Quantitative analysis of the kava lactones 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 being used.

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