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Research paper

Comparison of kava (*Piper methysticum* Forst.) varieties by UV absorbance of acetonic extracts and high-performance thin-layer chromatography

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ABSTRACT

The development of quality standards to regulate the trade of kava (*Piper methysticum*) in the Pacific necessitates new analytical techniques for routine control of high-quality raw material. In the present study, varieties and parts of the plant were compared for their kavalactone and flavokavin (FK) profiles. Three hundred and eight samples corresponding to 25 noble, 25 two-days and five *wichmannii* varieties were collected from 203 different plants. Commercial samples were also analysed to test the technique. Overall, 353 samples were prepared (221 roots, 114 stumps, nine basal stems and nine stem peelings). Their acetonic extracts were measured for UV absorbance and analysed by HP-TLC. Two ratios were computed after scanning the plates at 245 nm (K/KL = kavain/total kavalactones) and 366 nm (FK/KL = flavokavins/kavalactones). The results indicate that noble varieties suitable for daily consumption of kava are characterised by high K/KL and low FK/KL. At 400 nm, the mean UV absorbance of acetonic extracts from noble, two-days and *wichmannii* varieties are 0.69, 0.94 and 1.04, respectively. The significant (+0.757**) correlation between the total FK content and the extracts absorbance at 400 nm indicates that a simple colorimeter can be used to detect poor-quality raw material.

1. Introduction

Kava (*Piper methysticum* Forst.) is used to prepare the traditional beverage of the Pacific Islands by cold-water extraction of the underground organs of the plant. It is estimated that, in Fiji and Vanuatu, the two largest producing countries, approximately 25,000 ha are cultivated resulting in the production of 10,000 tonnes with 1100 tonnes being exported every year (Siméoni and Lebot, 2014). The Pacific Island countries are attempting to develop kava standards that could be accepted by the FAO/WHO Codex Alimentarius Commission to regulate quality and trade within the region (FAO/WHO, 2014). There is, however, a need for further development of analytical techniques capable of identifying on a routine basis the useful chemical components of the kava plant (kavalactones), but also undesirable molecules (alkaloids and flavokavins [FK]). The objective is to control the raw material for traditional kava beverage preparation for consistency and high quality, taking into account varieties, plant parts and geographical origins to encourage better traceability (Teschke et al., 2011a,b).

Morphological, chemical and genetic evidence and data suggest that *P. methysticum* derives from the wild species *P. wichmannii* C. DC. The two taxa are now considered a single species, with the cultivated varieties identified as *P. methysticum* var. *methysticum* and the wild forms as *P. methysticum* var. *wichmannii* (Applequist and Lebot, 2006). Wild kava exists in Papua New Guinea, the Solomon Islands and Vanuatu, while cultivated varieties are distributed throughout the Pacific from Vanuatu to Hawaii. The kavalactone content depends on the variety, organ (roots, stumps or basal stems) and age of the plant (usually harvested after three years of growth), as well as the environment (soil fertility and sunshine hours) (Wang et al., 2013). In Vanuatu, kava varieties are classified into three different groups, called “nobles”, “two-days” and “*wichmannii*”, based on the physiological effects of the beverage, which depend largely on the levels of six major kavalactones. Noble varieties are safe for daily drinking and are rich in kavain (K). Two-days and *wichmannii* varieties are low in K and rich in dihydrokavain (DHK) and dihydromethysticin (DHM). They are unsuitable for consumption and are known to cause nausea (Lebot and Lévesque, 1989).

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HPLC is the most commonly used analytical technique to identify kava chemotypes. This is done by coding the chemical composition of the extract in decreasing order of each of the six major kavalactones: 1 = demethoxyyangonin (DMY), 2 = dihydrokavain (DHK), 3 = yangonin (Y), 4 = kavain (K), 5 = dihydromethysticin (DHM), and 6 = methysticin (M). A good chemotype corresponding to a noble variety is coded 426153 or 243561 (Lebot and Lévesque, 1996). This simple system gives a comparatively good assessment of quality, but is subject to debate, as the results can vary according to the extraction solvent used (acetone, chloroform, ethanol, hexane or methanol). Acetone has been found to be the most effective solvent in terms of maximum yield and types of molecules extracted (Xuan et al., 2008). However, results can also vary according to the HPLC apparatus used, the type of column or the elution solvent. For a given noble variety, such as *borogu*, HPLC chemotypes can change from 423 to 246 or 245 (Siméoni and Lebot, 2002). Although these molecules have different absorption maxima, HPLC quantitation of the six major kavalactones is often carried out with a scan at a single wavelength varying from 220 nm (Shao et al., 1998; Denery et al., 2004), 240 nm (Boonen et al., 1997; Häberlein et al., 1997; Meissmer and Häberlein, 2005; Bilia et al., 2005), 245 nm (Gaub et al., 2004a), 246 nm (Ganzera and Khan, 1999; Shäfer and Wintehalter, 2005) to 254 nm (Smith et al., 1984).

Alkaloids have been isolated from *P. methysticum* leaves and stem bark (peelings) (Smith, 1979; Dragull et al., 2003), and pipermethystine has been suspected to be hepatotoxic (Lim et al., 2007). When the traditional beverage is prepared, these parts of the plant are never used, but with the increase in demand and the development of commercial plantations, post-harvest operations are not always properly conducted and controlled, and it is possible that these by-products are adulterating the final commercial powders. Also, peelings are considered a cheap source of kavalactones and have been purchased by extractors in the past (Teschke et al., 2011b); this unscrupulous trade still continues today.

FK have been documented as presenting anti-cancer properties (Ji et al., 2013; Zi and Simoneau, 2005), but they are also suspected to be potentially hepatotoxic (Jhoo et al., 2006; Zhou et al., 2010; DiSilvestro et al., 2007; Tang et al., 2010). FK are chalcones and pigments, and are easily detected through visual inspection of the fresh roots or in their solvent extracts, producing a bright yellow colour; however, some extracts have darker colours: amber or even brown. The content of flavokavins (FK) in the plant varies according to the variety. Tremendous variation also exists in FK content and resulting cytotoxicity levels of commercially available kava products found on the US market (Martin et al., 2014). FK are thought to be involved in the chemical composition of unsuitable chemotypes and must be analysed for quality control. They are easily detected with HPLC at 350 nm, and simultaneous detection of the major kavalactones and FK is feasible (Meissmer and Häberlein, 2005). However, because HPLC uses important volumes of solvents and is a fairly slow analytical technique (one sample per 30 min), it is not presently used for routine analysis in the Pacific producing countries. Near infra-red spectroscopy (NIRS) calibration equations have been developed for major kavalactones, but FK have not yet been analysed by NIRS (Gaub et al., 2004b; Gautz et al., 2006; Lasme et al., 2008; Wang et al., 2010). Recently, HP-TLC has been shown to be an efficient technique for the high-throughput routine detection of FK in kava (Lebot et al., 2014; Boudescoque et al., 2013; Hosu et al., 2015; Paramasivam et al., 2009).

The objectives of the present study were (i) to assess the potential of HP-TLC to differentiate kava varieties based on their kavalactones and FK profiles; (ii) to compare these profiles in different parts of the same plant (roots, stumps, stems); and (iii) to develop a simple test based on the absorbance of acetonic extracts to differentiate commercial products and detect those originating from undesirable varieties or presenting adulteration.

2. Materials and methods

2.1. Plant materials

All samples were collected from mature plants over three years of age. Three hundred and eight samples corresponding to 25 noble varieties, 25 two-days varieties and five *wichmannii* varieties were collected from 203 different plants grown: (i) in the germplasm collection of the Vanuatu Agricultural and Research and Technical Centre (VARTC) (152 plants) and ii) directly from farmers' fields (51 plants) in Santo Island (Table 1). In a previous study, DNA fingerprinting confirmed their classification into the three different groups of varieties (VandenBroucke et al., 2015). Three plants per variety representing each group (*borogu*, *palisi* and *wichmannii*) were also analysed for variation between roots (proximal and distal), stumps, basal stems (first two internodes) and bark peelings ($3 \times 3 \times 5 = 45$ samples) of the same plant. All samples were washed and cut into small cubic pieces of approximately 2×2 cm. Overall, 353 samples were prepared (221 roots, 114 stumps, nine basal stems and nine stem peelings) and then dried at 60–80 °C until they were a constant weight.

2.2. Sample preparation

Dry matter was ground into a kava powder with <2 mm particle size on Port-Vila, Efate Island, using a kitchen mill (Moulinex, France) (Lebot and Lévesque, 1989). Powder samples were stored at room temperature before solvent extraction. The kava powder was weighed and dried for 24 h in an oven at 65 °C to remove residual humidity (RH), as kava is highly hygroscopic (approx. 10–12% RH). Ten grams of powder were extracted overnight in 30 mL of acetone and sonicated for 30 min before centrifugation at 4500 rpm for 10 min. Part of the supernatant (5 mL) was then transferred to vials

Table 1
List of varieties: number of plants analysed for their roots (R) and stumps (S).

Nobles	R[a]	S	Two-days	R	S	*Wichmannii*	R	S
Ahouia	3	2	*Abogae*	3	2	*Maewo*	5	1
Borogou	18	6	*Abogae tabal*	3	2	*Meleliap*	4	1
Borogorou	2	2	*Birfock*	4	2	*Sini bo*	3	1
Borogoru memea	3	2	*Bogania*	5	2	*Vambu*	3	1
Gimonlagakris	3	2	*Borogoru tabal*	2	2	*Wichmannii*	5	2
Gorogor entepal	2	2	*Fabukhai*	4	2			
Kelai	1	1	*Fabularalara*	3	2			
Malamala	3	2	*Isa*[b]	5	2			
Malog lilab	2	2	*Laklak*	2	2			
Malog velablalas	5	2	*Malog rock*	4	2			
Malohuia	3	2	*Malogro*	2	2			
Malovuro	2	2	*Malogu bora*	1	1			
Melomelo	3	2	*Malogu bwagango*	2	2			
Ni kawa Pia	5	2	*Malovoke*	1	1			
Pade	5	2	*Nelimliun*	3	2			
Palarasul	1	1	*Palisi*	18	6			
Palasa	2	2	*Pirimerei*	3	2			
Puariki	1	1	*Ranapapa*	4	2			
Poua	2	2	*Ring*	1	1			
Raimelomelo	1	1	*Rongrongwul*	5	2			
Sese	1	1	*Sentender*	3	2			
Seselaralara	2	2	*Taritamaewo*	2	2			
Silese	2	1	*Tarivarus*	11	4			
Tafandai	3	1	*Tarivoravora*	4	2			
Vakorokoro	2	1	*Tudei*	11	2			
Total (n = 308)	77	46		106	53		20	6

[a] Samples collected in farmers' fields were lateral roots only to avoid the harvest and the destruction of the whole plant.
[b] Introduced from Papua New Guinea.

and stored in the refrigerator at 4 °C in the dark before HP-TLC analysis.

2.3. UV absorbance of acetonic extracts

The remaining supernatant of each sample acetonic extract (approx. 10 mL) was transferred to transparent test tubes (10 mm in diameter) and absorbance was measured using a WPA CO 7000 colorimeter (Biochrom Ltd., Cambridge, England) at ten different wavelengths (400, 440, 470, 490, 520, 550, 580, 590, 680 and 700 nm). A reference sample (acetone used as blank) was measured between each single wavelength measurement. When a sample reached an absorbance of more than 1.99, it was diluted and measured again. Acetonic extract samples representing each group of varieties (nobles, two-days and *wichmannii*) were also measured every nm from 400 to 500 nm with a Genesys 10S UV/vis spectrophotometer (Thermo Fisher Scientific, New Zealand) in quartz cell types (10 mm) of 3.5 mL to identify their peaks of absorbance (Lasme et al., 2008).

2.4. Standards

Methysticin, dihydromethysticin, kavain, dihydrokavain, yangonin and demethoxyyangonin were purchased from Sigma–Aldrich (Fluka, St Quentin Fallavier, France). FK A, B and C were obtained from Pr. H. Häberlein (Institute of Physiological Chemistry, Bonn, Germany). Second sets of FK A and B were purchased from LKT Laboratories Inc. (St Paul, MN, USA). Pipermethystine (alkaloid) standard was obtained from Pr. H.C. Bittenbender (University of Hawaii, Honolulu, HI, USA). Standard stock solutions were prepared by dissolving the appropriate amount of each standard molecule in acetone (1.0 mg/mL). Stock solutions were stored at 4 °C in the dark and were stable for several weeks. Standard solutions were applied on plates to locate the exact position (R_f values) of the nine molecules of interest. Peak purity tests were done by comparing UV spectra of the six individual kavalactones and three FK in standard and sample tracks. For the determination of the linearity curve, different amounts of stock solutions (0.1, 0.2, 0.4, 0.6, 0.8, 1.0 μL) of the six kavalactones and three FK were applied on HP-TLC plates and scanned at 245 and 366 nm. The calibration plots of peak areas versus concentrations were linear for all standards. Response factors were determined for each standard with R^2 coefficients all deemed acceptable above 0.99.

2.5. High-performance thin layer chromatography

The protocol was identical to the one used for the detection of FK (Lebot et al., 2014). Solvents (acetone, dioxane, hexane and methanol) and anisaldehyde reagents were from Sigma–Aldrich. Analyses were performed with Merck (Darmstadt, Germany) silica gel 60 F_{254} plates (20 × 10 cm), using a Camag (Muttenz, Switzerland) HP-TLC system equipped with an automatic TLC sampler (ATS4), an automatic developing chamber (ADC2), a visualiser and a TLC scanner 4 controlled with winCATS software. Standards and sample solutions were applied band wise (band length of 8 mm, 250 nL/s delivery speed, track distance 8.0 mm and distance from the edge of 15 mm). Plates were developed with hexane:dioxane (8:2 v/v) as the mobile phase (migration distance of 85 mm) at room temperature after 30 s plate pre-drying with 10 mL of mobile phase but no tank saturation. Visual inspection and documentation of the plates were done at 254 nm and 366 nm. Plates were scanned twice under the following conditions: scanning mode, reflectance mode at 245 nm and at 366 nm, D2 and W lamp slit dimension 8.00 mm × 0.20 mm, scanning speed 20 mm/s, data resolution 100 μm/step. Peak area measurements (expressed in area units, AU at 245 nm and 366 nm) were used to compute ratios of kavain/total kavalactones (K/KL) and flavokavins (A + B + C)/yangonin + desmethoxyyangonin (FK/KL) (Lebot et al., 2014).

2.6. Derivatisation

In order to have a better visualisation of all the bands present, selected plates were derivatised after development. An anisaldehyde-sulfuric reagent was prepared by adding carefully 10 mL of sulphuric acid to an ice cooled mixture of 170 mL methanol and 20 mL acetic acid. To this solution, 1 mL of anisaldehyde reagent was added. The reagent was colourless and was stored in the refrigerator at 4 °C before use. The plate was immersed in the reagent at a speed of 3 cm/s and an immersion time of 1 s using a TLC Chromatogram Immersion Device. The plate was then heated for 3 min at 100 °C and evaluated under white light.

2.7. Validation of the technique

To assess the robustness and the possible variation of the HP-TLC ratios (K/KL and FK/KL) within samples, two types of tests were conducted: (i) one sample of *borogu* and one of *wichmannii* roots coming from the same plant were divided into ten subsamples and analysed to evaluate intra-samples variation, and (ii) two commercial kava powders were purchased from a local supermarket, divided into 20 subsamples each, and analysed to compare the results.

2.8. Statistical analyses

Statistical analyses were performed using XLStat software (Excel, Addinsoft Company, Microsoft Corporation, New York, NY, USA) for linearity curves, principal components analysis (PCA) using Spearman coefficients of correlation and descriptive statistics on analysed samples.

3. Results and discussion

3.1. Absorbance of acetonic extracts

The different colours of the acetonic extracts obtained from different kava varieties are presented in Fig. 1. The results of the absorbance measurements recorded with the colorimeter at ten different wavelengths are presented in Table 2. Acetonic extracts from noble varieties (n = 123) presented very low mean

Fig. 1. From left to right, acetonic extracts of noble (test tubes 1–4), two-days (5–9) and *wichmannii* varieties (10–12) of *Piper methysticum* Forst.

Table 2
Mean acetonic extract absorbance of roots and stumps of nobles, two-days and *wichmannii* varieties measured with colorimeter, with K/KL and FK/KL ratios and sum of FK (A + B + C) content (AU) measured by HP-TLC.

Varieties	Absorbance of acetonic extracts at:										K/KL 245 nm	FK/KL 366 nm	FK AU
	400	440	470	490	520	550	580	590	680	700			
Nobles (*n* = *123*)	0.69	0.42	0.30	0.17	0.12	0.09	0.07	0.05	0.04	0.02	1.18	0.32	5387
SD	0.2	0.3	0.2	0.1	0.1	0.1	0.1	0.0	0.1	0.0	0.12	0.08	792
CV%	28.2	75.5	79.1	87.9	88.5	85.4	82.2	92.8	147.0	128.4	10.2	26.3	14.7
Two-days (*n* = 159)	0.94	0.84	0.61	0.33	0.22	0.15	0.11	0.08	0.05	0.03	0.54	1.02	21805
SD	0.1	0.5	0.4	0.3	0.2	0.1	0.1	0.1	0.0	0.0	0.066	0.32	4121
CV%	14.5	54.1	64.0	81.1	86.2	86.0	84.4	85.5	92.5	106.2	12.3	31.2	18.9
Wiichmannii (*n* = 26)	1.04	1.30	1.05	0.68	0.52	0.38	0.26	0.17	0.08	0.06	0.17	0.66	13329
SD	0.1	0.5	0.5	0.4	0.4	0.3	0.2	0.1	0.1	0.1	0.025	0.32	2999
CV%	12.5	41.2	50.1	66.0	74.3	74.5	76.6	85.3	99.6	112.5	14.8	48.2	22.5

absorbance, decreasing rapidly from 400 to 470 nm (0.69, 0.42, 0.30). Extracts from two-days varieties (*n* = 159) presented higher mean absorbance, decreasing slowly from 400 to 470 nm (0.94, 0.84, 0.61). Extracts from *wichmannii* varieties (*n* = 26) presented much higher mean absorbance, increasing from 400 to 440 nm and decreasing after (1.04, 1.30, 1.05). For the three groups of varieties, measurements taken at 400 nm were consistent with low standard deviations of their means (respectively, 0.2, 0.1, 0.1) and low coefficients of variation (respectively, 28.2%, 14.5%, 12.4%). Measurements taken with wavelengths above 500 nm were within the same proportions, with the nobles absorbing less than the two-days and *wichmannii* varieties (Fig. 2A). However, their means presented high standard deviations, indicating the weakness of measurements taken at such high wavelengths. This was further explained by the absorbance curve measured every nm with the spectrophotometer, which indicated no absorbance after 500 nm (Fig. 2B).

3.2. Absolute absorption spectra of kavalactones and FK

The calibration plot of peak area versus concentration was polynomial. For the nine standards, R^2 coefficients were very high. Individual standards of the nine compounds of interest were applied on plates and scanned after elution to obtain their UV spectra (Fig. 3). It appeared that there were two groups of compounds: those that could be easily detected and quantified around 245 nm (DHM, DHK and K) and those that were better detected around 360 nm (M, DMY, Y, FKB, FKA and FKC). The plates were, therefore, scanned twice. First, the plates were scanned at 245 nm to compute a ratio (K/KL) corresponding to the peak area of K versus the sum of the peak areas of all other kavalactones (DHM + DHK + M + DMY + Y). The same plate was then scanned at 366 nm and a second ratio was computed (FK/KL), corresponding to the sum of the peak areas of the three FK (A + B + C) versus the peak areas of Y and DMY following the HP-TLC protocol previously described for FK (Lebot et al., 2014). The results are presented in Table 2. Noble varieties were characterised by high K/KL (1.18 ± 0.12), low FK/KL (0.32 ± 0.08) and low total FK (5387 AU ± 792). Two-days varieties were characterised by low K/KL (0.54 ± 0.066), very high FK/KL (1.02 ± 0.32) and very high FK (21805 AU ± 4121). Finally, *wichmannii* varieties were characterised by very low K/KL (0.17 ± 0.025), high FK/KL (0.66 ± 0.32) and high total FK (13329 AU ± 2999) (Table 2).

3.3. High-performance thin-layer chromatography

Ten subsamples of roots coming from the same plant of *borogu* (noble) and ten coming from a *wichmannii* plant were compared for variation of their K/KL and FK/KL ratios. There was variation between subsamples within the same plant, but it remained acceptable for *borogu* and *wichmannii* (Table 3). Also, the mean values of the two ratios for these ten subsamples clearly differentiated the noble variety (1.06 and 0.29) and the *wichmannii* variety (0.16 and 0.48), which confirmed that these ratios were

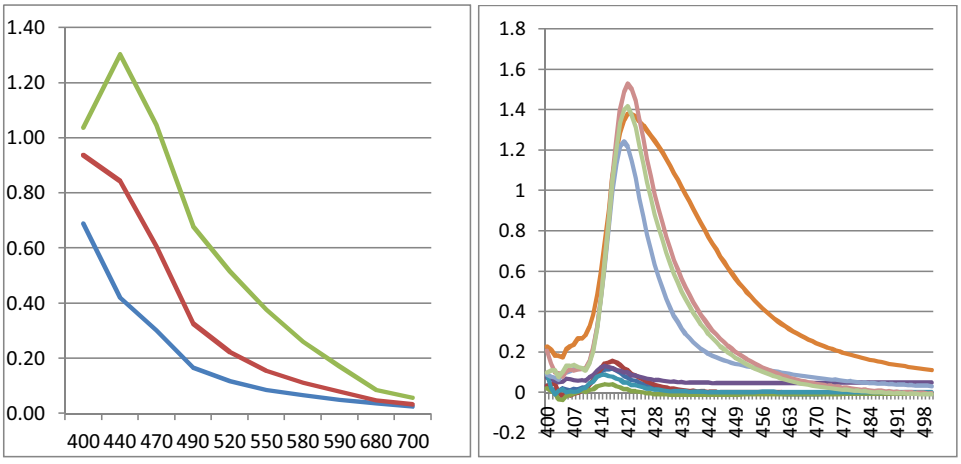

Fig. 2. (A) Mean absorbance of 123 extracts of nobles (blue), 159 two-days (red) and 26 *wichmannii* (green) at ten different wavelengths (measured with a colorimeter). (B) Absorbance every nm between 400 and 500 nm (measured with a UV/vis spectrophotometer). The noble varieties present very low absorption (around 410–420 nm), while two-days (blue, green, purple) and *wichmannii* (brown) varieties present peak absorption at these wavelengths. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

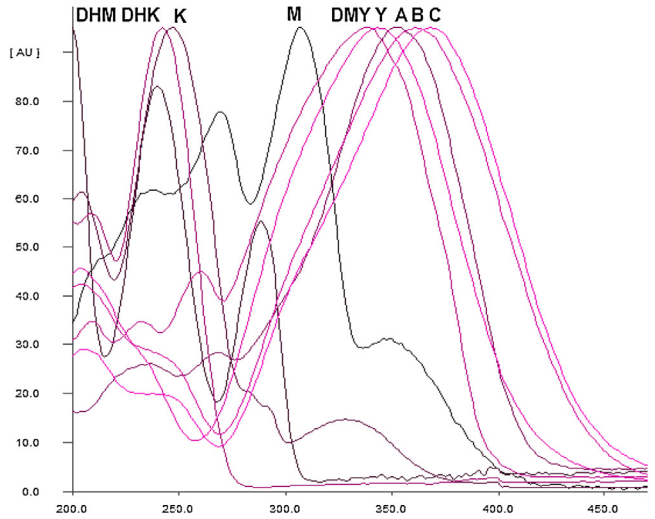

Fig. 3. From left to right, absorption maxima of DHM = dihydromethysticin (λ_{max} 200 nm), DHK = dihydrokavain (λ_{max} 242 nm), K = kavain (λ_{max} 247 nm), M = methysticin (λ_{max} 306 nm), DMY = desmethoxyyangonin (λ_{max} 338 nm), Y = yangonin (λ_{max} 354 nm), FKB = flavokavin B (λ_{max} 343 nm), FKA = flavokavin A (λ_{max} 361 nm) and FKC = flavokavin C (λ_{max} 368 nm).

discriminant and stable within the plant. Variation within two commercial products is presented in Table 4. The variation between sub-samples for K/KL and FK/KL ratios was acceptable for both products. However, the absorbance values of the acetonic extracts revealed significant variation above 490 nm, confirming that higher wavelengths were not useful to characterise and differentiate acetonic extracts.

A PCA was conducted on the data matrix (308 individual plant samples × 8 variables) (Fig. 4), and the results of the Spearman coefficients of correlation are presented in Table 5. Most correlation coefficients were statistically significant ($p > 0.01$). K/KL values (measured at 245 nm using densitometric scanning of TLC plates) were negatively and significantly correlated with FK/KL values (measured at 366 nm using densitometric scanning of TLC plates) and the FK (A + B + C) content (AU) measured by densitometry at 366 nm. Noble varieties presented very low absorbance of their acetonic extracts (measured at 400 nm), and these low values were correlated with low FK content. Very high correlations between values measured at shorter wavelengths (400–470 nm) and higher ones (>490 nm) indicated that there was no need to measure the samples at these higher wavelengths (Fig. 2A).

3.4. Variation between different parts of the plant

The HP-TLC profiles of variety representatives of the three groups (nobles, two-days, *wichmannii*) were compared for different parts of the plants (proximal and distal roots, stumps, basal stems and stem peelings). The results are presented in Fig. 5. For the same variety, no significant differences were found between proximal or distal roots, stumps and basal stems for the two ratios,

Table 3
Variation between ten subsamples of the same root samples originating from single plants of *Borogu* (noble variety) and *Wichmannii* varieties.

Borogu ($n = 10$)	K/KL	FK/KL	*Wichmannii* ($n = 10$)	K/KL	FK/KL
Mean	1.06	0.29	Mean	0.16	0.48
SD	0.06	0.012	SD	0.006	0.007
CV%	5.66	4.31	CV	3.75	1.46

but the basal stems presented lower total contents (tracks 4, 10 and 15). However, the stem peelings (tracks 5, 11, 16) of nobles, two-days and *wichmannii* all presented high FK/KL ratios. They also presented K (red band on Fig. 5B under white light) and other kavalactones, confirming their interest for potential importers and local traders. They are unsuitable for daily consumption and are an illegal by-product, but are a cheap source of kavalactones for unscrupulous buyers or for adulteration. Because of their peaks of absorbance (Fig. 3), FK were better scanned and quantified at 366 nm and were better visualised under white light. When scanned at 366 nm, they produced black bands difficult to detect visually (Lebot et al., 2014). Visualisation under white light (Fig. 5B) indicated that two-days and *wichmannii* varieties presented the upper yellow band corresponding to FKB. There was an extra yellow band in the *wichmannii* variety (indicated by FK in Fig. 5B), which appeared very similar to other FK. Scanning of all samples at 366 nm detected much higher levels of all FK (A + B + C) in two-days than in *wichmannii* and noble varieties, confirming previous results (Table 2) (Lebot et al., 2014).

Visualisation at 366 nm (Fig. 6) also indicated that extracts of peelings presented additional red bands that were absent in roots and stumps of nobles, two-days and *wichmannii* varieties. The application of a pure standard of pipermethystine (from Hawaii) on the plate confirmed that these bands do not correspond to the alkaloids detected in *P. methysticum* stem peelings, which have been identified in previous studies as pipermethystine and awaine (Dragull et al., 2003). In the absence of other pure standards, it was not possible to identify these molecules. They, however, represented very useful markers to detect the presence of peelings in kava powders. The alkaloid pipermethystine was considered a possible cause for alleged hepatotoxicity of kava-based products in Germany (Teschke et al., 2011a), but a comprehensive investigation conducted on a series of retained samples of German products concluded that it was absent (Lechtenberg et al., 2008). Our peeling extract profiles presented at least six compounds (red bands in Fig. 6), which were probably undesirable for the consumer. Their identification could contribute to elucidating the enigma of hepatotoxicity claims in Europe (Teschke et al., 2011b).

3.5. Differences between and within groups of varieties

Within each group of varieties, the chemical profiles amongst varieties of kava revealed with the two ratios (K/KL and FK/KL) were similar. Between the groups, the same ratios clearly differentiated the two-days and *wichmannii* varieties that were unsuitable for consumption from noble varieties. DNA

Table 4
Comparison of the variation between and within two commercial products of commercial kava powder (20 subsamples).

Sub-samples	K/KL	FK/KL	400	440	470	490	520	550	580	590	680	700
Mean product 1	1.16	0.27	0.48	0.22	0.17	0.09	0.08	0.06	0.06	0.07	0.06	0.06
SD	0.035	0.01	0.08	0.06	0.05	0.05	0.05	0.04	0.04	0.06	0.05	0.06
CV%	3.02	2.85	14.57	29.37	29.57	49.35	59.62	67.46	72.31	84.81	91.83	100.19
Mean product 2	1.11	0.36	0.57	0.22	0.20	0.10	0.10	0.09	0.09	0.07	0.06	0.06
SD	0.045	0.01	0.12	0.05	0.05	0.05	0.06	0.05	0.04	0.04	0.04	0.04
CV%	4.12	2.24	24.36	21.39	27.02	52.94	60.76	58.94	44.74	60.96	60.90	77.00

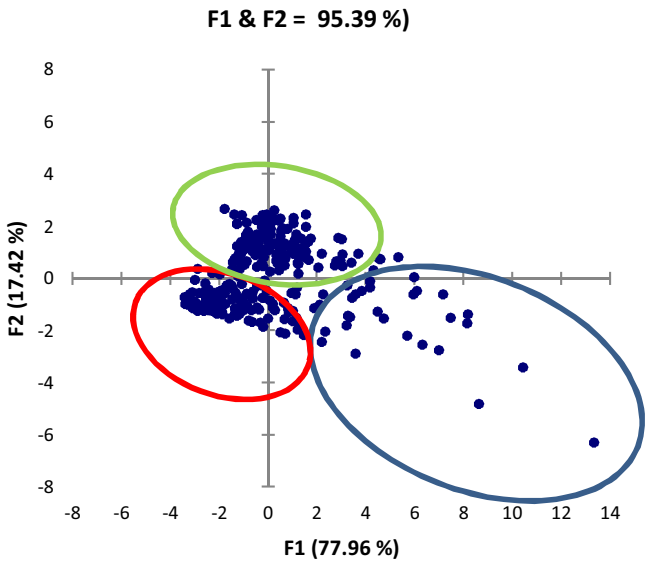

Fig. 4. Principal component analysis conducted on data matrix 308 individuals × 8 variables (K/KL, FK/KL and first six wavelengths recorded with colorimeter). The absorbance of the acetonic extracts are separated by axis F1 (77.96%) with low absorbance on the left and high absorbance on the right. The values of the two HP-TLC ratios (axis F2) are separating the noble varieties (red) from the two-days (green) and *wichmannii* (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

fingerprinting has been used to analyse the genetic diversity of kava varieties by combining two different sets of molecular markers (SSR, Simple Sequence Repeats and DArT, Diversity Array Technology). It was observed that noble varieties from different islands clustered together within a very narrow genetic base despite their morphological variation and that they evolved by the predominance of clonal selection. DNA fingerprints clearly differentiated the two-days and w*ichmannii* varieties revealing that they both clearly belonged to different genotypes (Vanden-Broucke et al., 2015). Each of these three groups of varieties encompassed distinct morphotypes when differentiated with morphological descriptors (Lebot and Lévesque, 1989), but they all belonged to the same genotype when discriminated with molecular markers. It was thus suggested that such low intra-group genetic diversity revealed that each group corresponded to a single clonal lineage. The occurrence of distinct varieties (morphotypes) sharing a common chemical profile (K/KL and FK/KL) confirmed that varieties within groups (i.e. nobles, two-days, *wichmannii*) were selected following clonal generations. Indeed, farmers from the Pacific are known to favour the selection of novel

Table 5
Spearman correlation coefficients (308 samples × 8 variables) (measurements above 520 nm are all highly correlated with the previous wavelength).

Variables	K/KL	FK (AU)	FK/KL	400	440	470	490
AU	−0.797**						
FK/KL	−0.848**	0.817**					
400	−0.530**	0.757**	0.612**				
440	−0.394**	0.747**	0.614**	0.972**			
470	−0.334**	0.718**	0.598**	0.958**	0.972**		
490	−0.252*	0.661**	0.549**	0.924**	0.940**	0.954**	
520	−0.177*	0.589**	0.479**	0.870**	0.889**	0.909**	0.934**

* Significant at $p > 0.05$.
** Significant at $p > 0.01$.

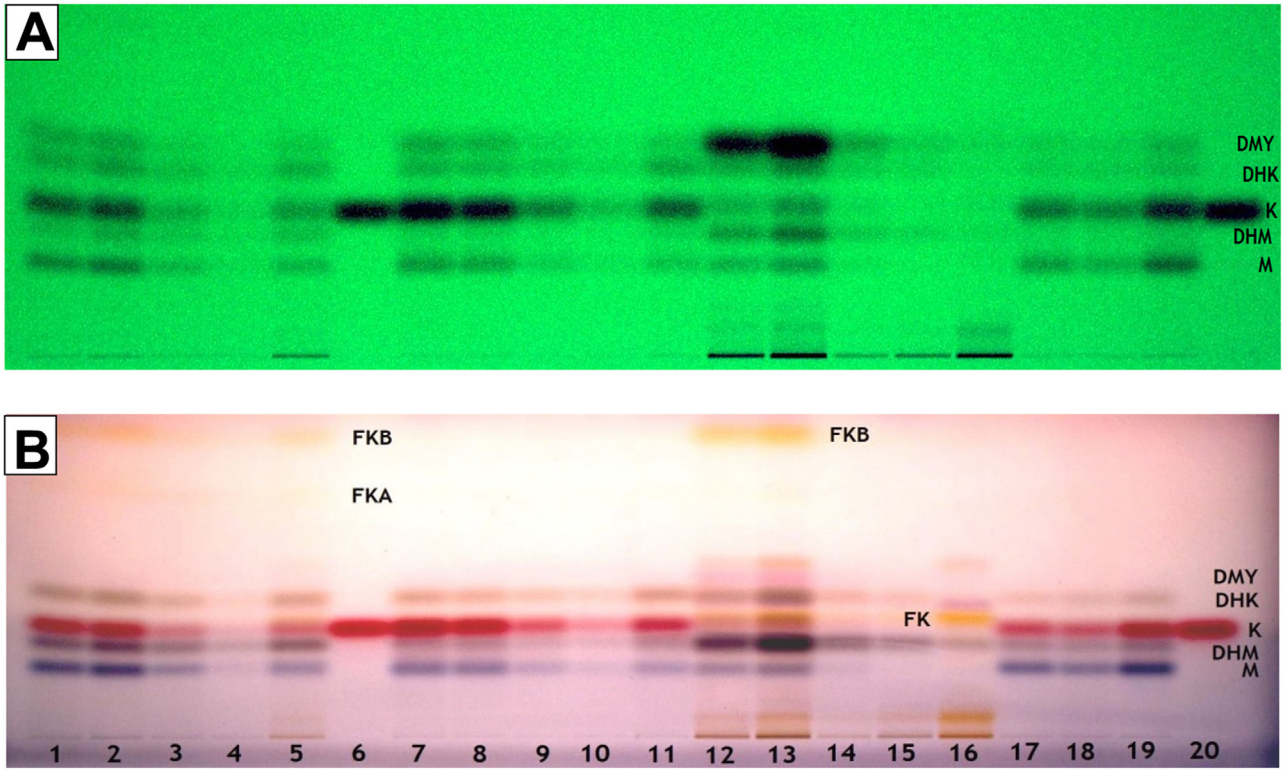

Fig. 5. Comparison of HP-TLC profiles for different plant parts and varieties. The upper plate (A) is visualised at 254 nm, the lower plate (B) is visualised with white light after derivation (anisaldehyde reagent). Tracks 1–5 correspond to acetonic extracts of proximal roots, distal roots, stumps, basal stems, and peelings of two-days variety *palisi*. Standard of pure kavain is inserted into tracks 6 and 20. Tracks 7–11 correspond to extracts of noble variety *borogu* (same order) and tracks 12–16 to *wichmannii* variety. Tracks 17–19 correspond to three noble varieties (*kelai*,*palarasul* and *sese*) used as controls. The upper yellow bands on plate B correspond to FKB (present in *palisi* and *wichmannii*, but absent in *borogu* and the three noble checks on tracks 7–11 and 17–19). *Wichmannii* presents an extra yellow band, similar to other FK. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

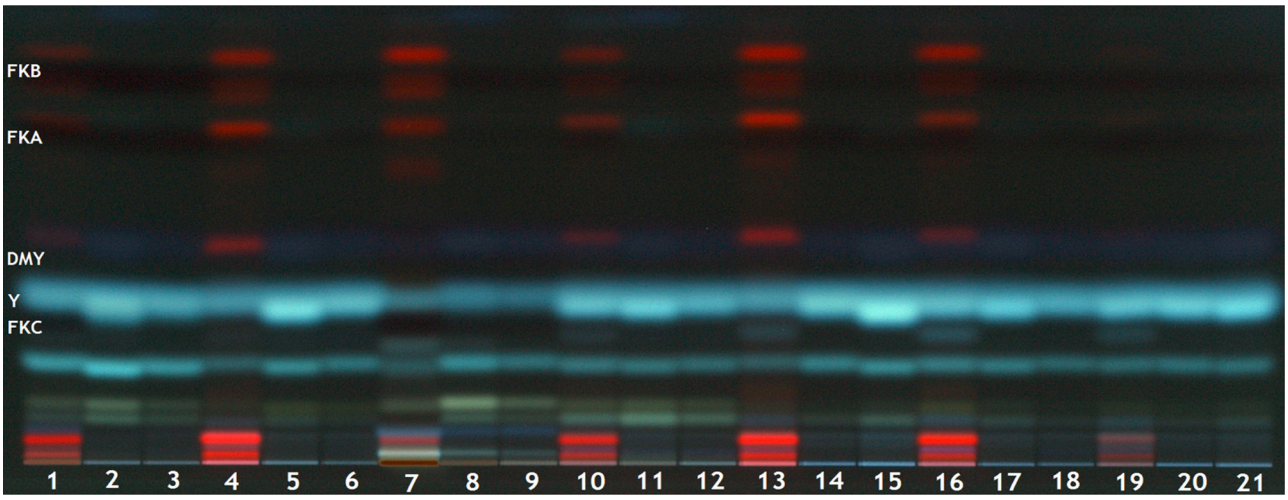

Fig. 6. Comparison of HP-TLC profiles scanned at 366 nm of varieties *palisi* (two-days), *borogu* (noble), *Sini bo* (*wichmannii*), *pia* (noble), *silese* (noble), *puariki* (noble), *kelai* (noble). Stem peelings (tracks 1, 4, 7, 10, 13, 16 and 19) are followed by stump and root extracts of the same plant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

variants and insert them into their varietal portfolio. This approach has been observed in other vegetatively propagated crops (Lebot, 1992), and this selection process is still dynamic and efficient in kava, where new morphotypes are regularly found by farmers. Clonal selection produced remarkable morphological traits (e.g. purple stems with round leaves vs. green stems with elongated leaves), but the ratios of K/KL and FK/KL were much more stable within each group, which might suggest that they were controlled by different sets of genes. For plants, such as kava, used intensely for the preparation of a daily traditional beverage, morphological characters are selected by farmers for the shear sake of having diversified portfolios, but are less important than chemical profiles. The selection pressure remains on the chemical composition. The chemotype with high K/KL and low FK/KL is responsible for the quality of the effects while the total kavalactone contents determine its intensity, but it has been shown to vary according to the variety and environmental factors (Wang et al., 2013) Important variations in total kavalactones between and within groups of varieties have already been documented (Lasme et al., 2008) and may be responsible for the variation observed for K/KL and FK/KL in the present study (Table 2).

3.6. Potential applications for routine analysis

DNA fingerprinting could be useful for quality control, but is expensive, laborious, not high-throughput, and necessitates access to fresh leaves for good quality DNA extraction. However, once the plant has been harvested and the stems and leaves have been removed, it is impossible to distinguish kava varieties, as the product is traded under the form of fresh stumps in the local market and as sun-dried roots or chips for export. Quality standards are, therefore, difficult to apply without the chemical analysis of numerous samples (VandenBroucke et al., 2015).

A useful result of the present work was the detection of clear chemical differentiation between high- and low-quality varieties from Vanuatu. Previous studies involving field experiments and analyses of kavalactones have already shown the stability of chemotypes across locations for a given variety and confirmed that chemotypes are genetically controlled. However, this study enabled chemical discrimination of numerous varieties differing in their HP-TLC ratios and assessed though major quality-related traits: the K and FK contents. These results are also consistent with genetic data by differentiating the noble varieties suitable for daily consumption from the unsuitable two-days and *wichmannii* varieties (VandenBroucke et al., 2015). These two groups of kava varieties may not be sold in Vanuatu (Kava Act, 2002), but two-days varieties are favoured by young farmers because they are fast growing and high yielding. There is an urgent need to enforce the existing legislation by developing the efficient control of the raw material using high-throughput analytical techniques.

NIRS calibrations for compounds, such as FK and alkaloids, present in very small quantities in the raw material, might be difficult to develop, but further research is needed. HPLC is the most frequently used analytical technique for kavalactones (Bilia et al., 2005), FK (Meissmer and Häberlein, 2005) and alkaloids (Dragull et al., 2003). Previous HPLC studies attempting to characterise kava extracts by quantitation of the six major kavalactones used only one wavelength from 220 nm to 254 nm. There is, however, a technical constraint with the choice of these wavelengths. Quantitation would be accurate only for DHK (λ_{max} = 242 nm) and K (λ_{max} = 247 nm). DHM, M, DMY and Y have a λ_{max} of 200 nm, 306 nm, 338 nm and 354 nm, respectively, and FK B, A and C have a λ_{max} of 343 nm, 361 nm and 368 nm (Fig. 3). It is, therefore, necessary to scan the same extract sample at least at two different wavelengths, but with HPLC, this adds extra time per sample. HP-TLC appears to be a cost-efficient, eco-friendly, analytical technique for kava as about 20 tracks (samples and standards) can be analysed on a single silica plate with only 10 mL of solvent. The same plate can be scanned twice, first at 245 nm for the K/KL ratio and then at 366 nm for the FK/KL ratio, giving (in less than one hour) a reliable assessment of the quality of these 20 samples, as the two ratios are negatively correlated.

Over the years, numerous studies have shown that the major factor influencing the quality of the kava beverage is the variety (Smith, 1979; Lebot and Lévesque, 1989; Wang et al., 2013), but adulteration has also been a constant problem in the South Pacific (Duve and Prasad, 1981), and there is a need for efficient routine control (Duve and Prasad, 1983; Teschke et al., 2011b). An unexpected result of the present study was the detection of useful markers that could be used to control the illegal use of stem peelings, often used for adulteration in kava powders. There is a need for further research to identify the different molecules involved in the dark colours of the extracts (most likely a combination of numerous different pigments and tannins). However, the use of a simple colorimeter, such as the one tested in the present study, allows for fairly easy control of the raw

material quality with comparatively affordable equipment, which is very encouraging for the development of standards throughout the Pacific region; nonetheless, a more comprehensive survey is necessary to characterise varieties from Fiji, Tonga, Samoa, Hawaii, Pohnpei and Papua New Guinea.

In the early 2000s, kava-based products manufactured in Europe were suspected of hepatotoxicity; however, there was no evidence for such claims. It was shown that quality-control procedures were lacking in Europe and in the Pacific at that time (Teschke et al., 2011a). Two-day or *wichmannii* varieties rich in FK, or peelings rich in undesirable compounds, might have been involved, although it was never demonstrated that these suspicions were valid. In the near future, Codex Alimentarius type of standards for the traditional kava beverage will be obliged to address issues for quality standardisations such as kavalactone chemotypes in different varieties, the analysis of FK and alkaloids, and a definition of analytical protocols (Codex Alimentarius Commission E, 2010). Once established, it will be necessary to enforce the standards using a suitable analytical technique for routine control, and HP-TLC offers interesting perspectives for such controls.

4. Conclusions

HP-TLC offers interesting perspectives, such as cost-efficient, eco-friendly and high-throughput analytical techniques, for the routine analysis of kava raw material. The developed fingerprinting method is rapid and makes it possible to distinguish amongst noble, two-days and *wichmannii* varieties of kava based on their K/KL, FK/KL, FK contents (AU), and the UV absorbance of their acetonic extracts. These three distinct groups of chemotypes correspond to those identified by DNA markers as distinct clonal lineages and genotypes. Stem peeling extracts present several markers that make possible their detection in kava powders in case of adulteration. These results could therefore contribute to the enforcement of the Kava Act passed in the Vanuatu Parliament to regulate the industry in Vanuatu, and to routinely control high-quality kava raw material for both local and export markets.

Conflict of interest

The authors declare no competing financial interests.

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