*High-Throughput Analysis of Flavokawains in Kava (*Piper methysticum *Forst. f.) Roots, Chips and Powders and Correlations with Their Acetonic Extracts Absorbance*

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High-Throughput Analysis of Flavokawains in Kava (*Piper methysticum* Forst. f.) Roots, Chips and Powders and Correlations with Their Acetonic Extracts Absorbance

Vincent Lebot[1] · Juliane Kaoh[2] · Laurent Legendre[3,4,5,6]

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Abstract
Kava is a non-alcoholic beverage prepared by cold water extraction of the ground roots and stumps of *Piper methysticum* Forst. f. Kava contains flavokawains (FKs) which have been suspected of being potentially cytotoxic. Current HPLC protocols are not adapted to high-throughput quantification before export. The objectives of the present study were (i) to analyse with HPTLC the individual FKs in roots, stump, stems and peelings of four varieties grown in a controlled environment; (ii) to quantify FKs in 1053 commercial samples exported from Vanuatu in 2017-18-19 (370 roots, 381 chips and 302 powders) and (iii) to assess the efficiency of a colorimetric test for routine control. HPTLC plate scanning at 355 nm offered good linearity for three FKs with R^2 > 0.99 and RSD < 3.0%. High total FKs (> 14 mg/g DW) were found in poor-quality varieties and in peelings unsuitable for consumption. Plant parts known for their good quality, such as roots and peeled stumps of noble varieties, presented low total FKs (< 7 mg/g). Great variation was observed in exported roots (2.53–24.56 mg/g), chips (2.73–18.03 mg/g) and powders (2.92–16.41 mg/g). HPTLC proved reproducible for the high-throughput quantification of FKs in kava. A positive relationship was confirmed between the absorbance of the acetonic extract and the total FKs (R^2 = 0.5211) (n = 1053). Multivariate analyses revealed that in roots, chips and powders, the three FKs are significantly correlated with high absorbance values. The absorbance of the acetonic extract gives a fair assessment of the FK content in kava products.

Keywords Chalcones · Cytoxicity · Flavokawains · Kavalactones · HPTLC · Plant partitioning

Introduction

Kava, the non-alcoholic traditional beverage of the Pacific, is prepared by cold water extraction of the ground fresh or dried roots and stumps of the plant species *Piper methysticum* Forst. f. The effect of kava can be refreshing when it is diluted and relaxant when it is more concentrated. Clinical trials examining the efficacy of aqueous extracts of kava have not identified adverse health effects. The consumption of kava is considered safe and this beverage does not produce addiction or side effects when drank in moderation (FAO/WHO 2016). Dry kava is rich in starch (approx. 50% dry matter) and fibres (20%) with low minerals (3%), proteins (3%) and sugars (3%) and a residual humidity around 11%. For consumers, the most interesting compound is kavain, a kavalactone producing a fast relaxing effect (Chua et al. 2016). There are five other major kavalactones (KLs: methysticin, dihydromethysticin, dihydrokavain, yangonin and desmethoxy yangonin) and their composition and content are highly variable, depending on the age of the plant, the variety, the part of the plant and the environmental conditions (Wang et al. 2013).

During the last decade, the international trade of kava has expanded rapidly and it is estimated that approximately 6000 tonnes of dried roots, chips and powders are traded annually and the market is still growing (PHAMA 2015). The major producing countries are located in the Pacific (Fiji, Samoa, Tonga and Vanuatu) but kava is increasingly imported in the USA where it is sold on the Internet and in urban kava bars (Martin et al. 2014). The *Codex Alimentarius* of the FAO/

✉ Vincent Lebot
lebot@vanuatu.com.vu

[1] CIRAD, UMR-AGAP, PO Box 946, Port Vila, Vanuatu
[2] VARTC, PO Box 231, Luganville, Santo, Vanuatu
[3] Université de Lyon, F-69622 Lyon, France
[4] Université Lyon 1, Villeurbanne, France
[5] CNRS, UMR 5557, Ecologie Microbienne, Villeurbanne, France
[6] INRA, UMR 1418, Villeurbanne, France

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WHO is now developing regional standards to assist quality control (FAO/WHO 2019).

Kava also contains chalcones called flavokawains (FKs) and several studies have shown that these phenolic compounds belonging to the flavonoids family present cytoprotective properties and have potential against various diseases (Szliszka et al. 2010). FKA, B and C are the three chalcones pigments found in kava (Abu et al. 2013). FKA has an anti-inflammation activity (Kwon et al. 2013) and has been found to induce apoptosis of tumour cells and to possess anti-cancer properties (Zi and Simoneau 2005; Tang et al. 2008; Abu et al. 2014; Pinner et al. 2016; Hseu et al. 2018). FKB has cytotoxic and apoptotic effects against different types of cancer cells (Abu et al. 2016; Hseu et al. 2012; Ji et al. 2013; Li et al. 2011, 2014, 2019; Lin et al. 2012; Mohamad et al. 2010; Rossette et al. 2017; Tang et al. 2010; Yeap et al. 2017; Zhao et al. 2011). FKC is the least studied but has an anti-inflammatory effect (Park and Han 2016).

FKB has also been suspected of being potentially hepatotoxic (Jhoo et al. 2006; Teschke et al. 2011; Zhou et al. 2010). FKA and FKB can potentiate acetaminophen-induced hepatotoxicity and this observation suggested that kava and a drug interaction may account for potential hepatotoxicity (Narayanapillai et al. 2014). FKA and FKB were probably the major but not the sole compounds responsible for the ethanolic extracts toxicity of commercial kava imported in the USA while aqueous extracts showed no toxicity (Martin et al. 2014). The in vitro metabolism of FKA, B and C has also been characterised using human liver microsomes and it was concluded that the absolute amount of conjugated metabolites in vivo maybe even higher than the significant levels observed in vitro (Zenger et al. 2015). It has been suggested that FKs could be removed from kava aqueous extracts by isoselective focused adsorptive bubble separation (Backleh et al. 2003) but this would represent an extra cost to traded kava. The development of an efficient analytical technique for the determination of FKs appears better adapted as FKs are compounds that deserve greater attention to secure the safe international trade of kava (FAO/WHO 2016).

FKs can be quantitated using HPLC (Bilia et al. 2004; Meissmer and Häberlein 2005; Martin et al. 2014; Liu et al. 2018) but this technique is cumbersome and not convenient for high-throughput analysis of hundreds of samples before export from the Pacific. High-performance thin-layer chromatography (HPTLC) is increasingly used as a reliable analytical method for large-scale screening of samples within a short period, accompanied by unambiguous identification and quantification of compounds of interest (Wang et al. 2018). HPTLC has been shown to be reliable and cost efficient to differentiate good kava varieties suitable for daily consumption (called "nobles") from poor varieties producing hangovers (called "two days") and non-consumable wild kava (called "wichmannii") (Lebot et al. 2014; VandenBroucke et al. 2015). The differentiation was, however, based on FK/KL peak area ratios and individual FKs were not quantified. A colorimetric test based on the absorbance of the acetonic extract of kava powder has been shown to be useful to differentiate these three groups of kava (Lebot and Legendre 2016). But this test presents practical shortcomings because of the high absorbance of acetone below 340 nm, a value greater than the absorbance maxima of most KLs. The absorption of acetone extracts reflects the total quantity of KLs and FKs rather than their respective individual quantities. Diethyl ether was found to be more accurate than acetone for this colorimetric test (Lhuissier et al. 2017) but this solvent is in practice more difficult to use for routine analysis. The absorbance of the acetonic extract measured at 440 nm is nowadays used before export as a routine test to detect kava products suspected of being prepared from unsuitable varieties.

However, the pricing system presently used for the international trade of kava is based on the different parts of the plant and not on varieties. Dry kava is traded under the form of full roots, chips or powders. Roots are more expensive because they present higher KL content. This content decreases progressively from the roots to the stump (also called rootstock or rhizome) to the basal stems and to the upper stems (Smith 1983; Smith et al. 1984). Chips are prepared from sun-dried slices of peeled stumps (approx. 1 cm thick) and represent a lower grade but they account for almost 80% of the plant total commercial yield. Basal stems are of poor quality and are usually not traded but are often blended into commercial powders. Sales of stumps and stems peelings are illegal because these products are considered as unsuitable for consumption. They are sometimes used by dishonest traders for unscrupulous adulteration of ready-made powders (Duve and Prasad 1981).

The objectives of the present study are (i) to analyse with HPTLC the individual FKs in roots, stump, stems and peelings of four varieties grown in a controlled environment; (ii) to quantify FKs in 1053 commercial samples exported from Vanuatu in 2017-18-19 (370 roots, 381 chips and 302 powders) and (iii) to assess the efficiency of a colorimetric test for routine quality control before export.

Materials and Methods

Field Experiment and Plant Partitioning

Four different genotypes of kava (noble, two days, isa and wichmannii), representative of four genetic groups identified with DNA markers (VandenBroucke et al. 2015), were planted at VARTC (Vanuatu Agricultural Research and Technical Centre in Santo, Vanuatu (15° 23′ S and 166° 51′ E, ~ 80 m above sea level). Noble, two days and isa belong to cultivated kava (*P. methysticum* var. *methysticum*) and

wichmannii is a wild form belonging to *P. methysticum* var. *wichmannii*. As they were reproduced asexually through stem cuttings, all plants of each genotype were clones of the same mother plant and all plants were grown in a common field to minimise variation due to environmental factors. The plant spacings were 2 m between plants on the line and 2.5 m between the lines. For each variety, three different plants established the same day were harvested together after 3 years of growth when fully mature. Upon harvest, the twelve plants were partitioned in six different parts: distal roots, proximal roots (corresponding to two-halves of the lateral roots), peeled stumps, peeled basal stems, stumps peelings and stem peelings (Fig. 1), representing a total of 72 samples. All parts were washed by hand under cold running water and cut into small pieces with a knife. Fresh samples were oven-dried at 70 °C and dried fractions were sent to the Food Lab of the Department of Agriculture in Port Vila on Efate, Vanuatu.

Commercial Export Samples

Samples collected by the Department of Biosecurity officers (Ministry of Agriculture, Livestock, Forestry, Fisheries and Biosecurity, Vanuatu) during routine controls of exported kava for the years 2017 (n = 206), 2018 (n = 332) and 2019 (n = 515) were preserved in closed cartons in an air-conditioned room in Port Vila. The 1053 samples were then transferred to the Food Lab where the year of export and the type of product (roots, chips and powder) were recorded. All commercial samples were then oven-dried at 60 °C and were preserved into zip-lock bags kept in plastic boxes until analysis.

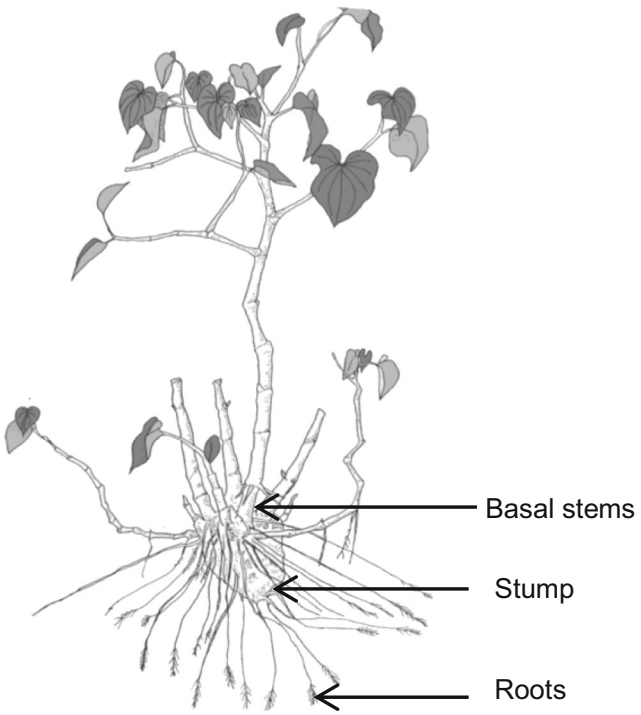


Fig. 1 The kava plants were partitioned into proximal and distal roots, peeled stump and basal stems, and stump and basal stems peelings

Chemicals and Standards

Analytical grade solvents (acetone, dioxane, hexane and methanol) were from Sigma-Aldrich. FKA, B and C were purchased from LKT Laboratories Inc. (St. Paul, MN, USA). Methysticin (M), dihydromethysticin (DHM), kavain (K), dihydrokavain (DHK), yangonin (Y) and desmethoxy yangonin (DMY) analytical grade standards were from Sigma-Aldrich (Fluka, France). Standard stock solutions were prepared by dissolving 1.0 mg of standard in 1.0 mL of acetone. Standard solutions were stored in the fridge at 4 °C and were stable for several weeks. Peak purity tests were done by comparing UV spectra of the three FKs and six KLs in standard and sample tracks following a previously described detailed protocol (Lebot et al. 2014). For the determination of the standards linearity curve, six different amounts of stock solutions (0.1, 0.2, 0.4, 0.6, 0.8, 1.0 μL) were applied on HPTLC plates and were scanned at 355 nm for FKA, B, C and Y and DMY and at 240 nm for the other four KLs: M, DHM, K and DHK.

Preparation of Kava Extracts

The dried samples were ground into a very fine powder using a coffee grinder (SEB, Prep'Line 850, Dijon, France). For each sample, 10 g of powder was transferred to a 50-mL polypropylene centrifuge tube (CellStar Tubes, Greiner Bio-One GmBH, Frickenhausen, Germany) and 30 mL of acetone was added. These tubes were sonicated in a water bath (Lab Companion UC-02, Cole Parmer, Vernon Hills, Il, US) for 10 min and then centrifuged at 1585×g for 10 min in a Universal 32 centrifuge (Hettich Zentrifugen, Tuttlingen, Germany). Finally, part of the supernatant was transferred to a 9-mm wide opening screw-thread vial of 2 mL in white glass (Chromacol™, Thermo Fisher™, USA) and stored in a refrigerator at 4 °C until analysis.

Colorimetric Test

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 440 nm. A reference sample (acetone used as blank) was measured between each sample.

High-Performance Thin-Layer Chromatography

All analyses were performed with Merck (Darmstadt, Germany) silica gel 60 F254 plates (20 × 10 cm), using a

Camag (Muttenz, Switzerland) HPTLC system equipped with an automatic TLC sampler (ATS 4), an automatic developing chamber (ADC 2), a visualizer and a TLC scanner 4 controlled with winCATS software. Standards and sample solutions were applied as bands (length of 8 mm, 250 nL/s delivery speed, track distance 8.0 mm and distance from the edge of 15 mm). The 10-mL mobile phase used to develop the plates was hexane:dioxane (8:2 v/v) with a migration distance of 80 mm at room temperature after 30 s of pre-drying and no tank saturation. Visual documentation of the plates was carried out at 254 nm and 366 nm. The plates were then scanned in reflectance mode at 240 nm (for M, DHM, K, DHK) and at 355 nm (for FKC, Y, DMY, FKA, FKB) with D2 and W lamp slit dimension 8.00 mm × 0.20 mm, scanning speed 20 mm/s, data resolution 100 lm/step. Peak area measurements (in area units, AU) were used. The total analysis time was 50 min for 20 samples applied on a single HPTLC plate with 10 mL of the mobile phase.

Statistical Analyses

Peak area measurements (in area units, AU) were compared with those of individual standards and corresponding values were translated into milligram per gram dry weight (DW). Raw data (peak areas and their transformation in %DW) were recorded using Excel™ (Microsoft Corporation) spreadsheet format. Statistical analyses were performed using ExcelStat software (Microsoft) for linearity curves of standards, normality of distribution tests and principal components analysis (PCA, Spearman coefficients of correlation, mean standard deviation and ANOVA Fisher's test of least significant difference (LSD) at $p \leq 0.05$).

Results and Discussion

Reproducibility Assessment

For the three FKs and six KLs, the repeatability of the measurements was assessed for each standard and the calibration plots equations (peak areas versus concentrations) are presented in Table 1. All were linear for the analytical standards with R^2 values > 0.99 ($p = 0.01$). The repeatability of the sample measurement of the peak area was assessed by injecting three replicates of the standard on the same day. The procedure was repeated over 3 days (three replicates each day) and the intra- and inter-day variations were assessed with relative standard deviation (%RSD). For each compound, %RSD values were low (< 3.0%) indicating that the HPTLC measurements are reproducible enough to be used for the quantification of individual compounds (Table 1). FKA, FKB and FKC peaks were well resolved when scanning the plates at 355 nm, along with Y and DMY (Fig. 2a). Scanning at 240 nm allowed the quantification of M, DHM, K and DHK. Chlorophyll pigments appeared as red bands at 366 nm and were easily detected when present in stem peelings (Fig. 2b). As expected, they were absent from underground organs (roots and stumps). The high-throughput analysis was made possible by applying 20 samples on a single HPTLC plate with 10 mL of solvent corresponding to 2.5 min and 0.5 mL of solvent per analysed sample (Fig. 3).

Table 1 Linearity of HPTLC measurements, accuracy and precision of repetitions (peak areas versus concentrations applied)

Compounds		Linear equation	R^2	% RSD
Measured at 240 nm				
Methysticin	M	$y = 11741x + 391.34$	0.9955	2.9
Dihydromethysticin	DHM	$y = 3833.2x + 343.29$	0.9965	1.1
Kavain	K	$y = 10960x + 2796$	0.9964	2.2
Dihydrokavain	DHK	$y = 6808.5x + 837.52$	0.9949	2.8
Measured at 355 nm				
Yangonin	Y	$y = 6976.2x + 676.76$	0.9943	2.6
Desmethoxy yangonin	DMY	$y = 11189x + 451.87$	0.9958	1.9
Flavokawain A	FKA	$y = 701.75x + 468.98$	0.9943	2.1
Flavokawain B	FKB	$y = 687.28x + 535.66$	0.9978	1.1
Flavokawain C	FKC	$y = 713.35x + 286.5$	0.9919	1.3

R^2, $p < 0.01$; *RSD*, repeatability standard deviation ($n = 5$)

Variation Between Different Parts of the Kava Plant

For the six different plant parts, corresponding to four distinct kava genotypes, the means of three individual plants analysed are presented in Table 2. In noble variety, the distal and proximal roots, stumps and basal stems presented low total FKs (respectively 5.79, 6.45, 6.12 and 3.62 mg/g). The two-day variety presented high total FKs (17.92, 20.62, 16.28, 6.99 mg/g). Isa, a variety known for its poor quality (10.61, 12.10, 10.41, 7.85 mg/g) and wichmannii, a wild form (14.12, 14.45, 13.89, 4.68 mg/g), presented intermediate total FKs values but these values were clearly higher than those of the noble variety. Interestingly, for the three cultivated varieties (noble, two days, isa), the total FKs were higher in the stump peelings (10.05, 23.57, 16.54), indicating that the plant is stocking FKs in the epidermal cells layers and that these compounds may have a protective role. Nevertheless, total FKs were lower in noble variety stem peelings (6.63 mg/g) than in two-day kava (19.72 mg/g) and isa (11.90 mg/g). Isa is a variety from Papua New Guinea, genetically distant from the noble and two-day varieties of Vanuatu (VandenBroucke et al. 2015) and known for its poor quality. Surprisingly, wild kava (wichmannii) has higher FKs in its roots and stumps (14.12, 14.45, 13.89 mg/g) than in its stump and stems peelings (11.69, 9.66 mg/g). Overall, the standard deviations of the means (± SD) were low for all different plant parts,

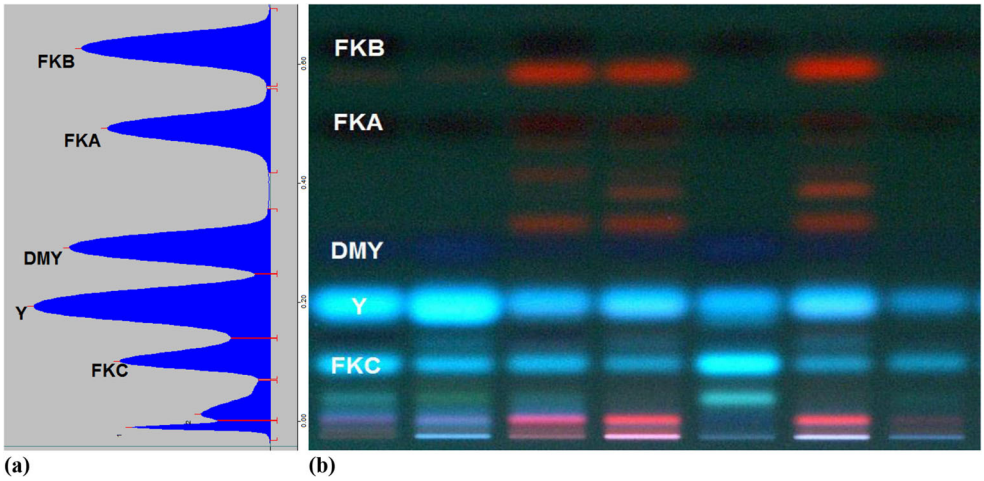


Fig. 2 **a** Chromatogram obtained at 355 nm showing peaks for flavokawain C (FKC), yangonin (Y), desmethoxy yangonin (DMY), flavokawain 1 (FKA) and flavokawain B (FKB). **b** Corresponding HPTLC plate at 366 nm

indicating that there is little variation between the three plants (clones) of the same variety. Based on these results, it appears that total FKs can be used as a criterion for poor-quality kava products because high FKs corresponded either to a poor-quality variety (two days, isa, wichmannii) or to plant parts (stump and stem peelings) known to be unsuitable for consumption. Suitable plant parts known for their high quality such as the roots and chips of peeled stumps of noble varieties presented very low total FKs (Table 2).

Acetonic extracts absorbance values (OD_{440}) for noble variety plant parts were low for distal, proximal roots, stumps and basal stems (0.39, 0.51, 0.23, 0.38). The absorbance values for two days (1.61, 1.31, 1.12, 0.66), isa (1.22, 1.31, 0.73, 0.52) and wichmannii (1.99, 1.84, 1.45, 0.91) were higher. Their extracts presented a characteristic amber colour for two days and isa, much darker than the light yellow colour of the noble variety plant parts. The wichmannii extracts were brown. Stump and stem peelings of the four varieties presented high values but were highest for two days and wichmannii (1.99 for both). These high absorbance values can be explained by the contribution of chlorophyll pigments appearing as red bands at 366 nm (Fig. 2b). However, the stumps are usually not exposed to light, so other non-identified pigments are most likely also involved. Linear regression analysis conducted on 60 samples corresponding to all plant parts of the four varieties, excluding stem peelings because of their very high absorbance biased by chlorophyll pigments, gave an R^2 of 0.5424 between OD_{440} and total FKs (Fig. 4). This R^2 reveals a positive relationship between the total FKs present in the different plant parts and their acetonic extract absorbance measured at 440 nm. It, therefore, appears that the higher is the total FKs in the extract and the higher is the absorbance value, although other pigments are probably contributing as well.

As expected, noble variety plant parts were rich in K with a mean content above 24 mg/g in the roots and up to 17.9 mg/g in the stump. The two-day variety presented high K in the roots and stumps (21.72, 23.46, 14.95 mg/g) but it also

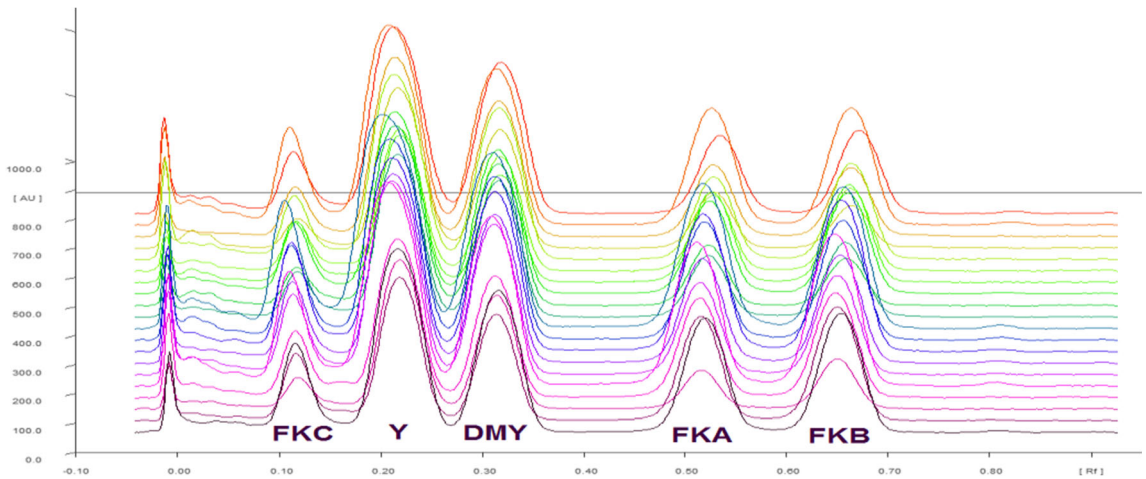


Fig. 3 HPTLC chromatograms measured at 355 nm for 20 tracks (samples) on a single plate showing well-separated peaks with from left to right: FKC, Y, DMY, FKA and FKB

Table 2 Mean contents ($n = 3$, mg/g) for flavokawains (FKs) and kavalactones (KLs) in different plant parts of kava (noble, two days, isa, wichmannii) and absorbance of their acetonic extracts (at 440 nm)

Varieties	Flavokawains				Absorbance	Kavalactones							FK/KL
	FKC	FKA	FKB	FKs ± SD		M	DHM	K	DHK	Y	DMY	KLs ± SD	
Noble													
Roots (distal)	1.68	1.82	2.29	5.79 ± 0.3	0.39	7.84	7.22	24.36	17.39	20.01	16.15	92.98 ± 3.6	0.06
Roots (proximal)	2.10	2.39	1.96	6.45 ± 0.9	0.51	7.97	6.17	24.78	18.24	22.17	16.92	96.25 ± 5.8	0.07
Stump	1.36	2.67	2.09	6.12 ± 1.2	0.23	4.22	6.72	17.92	12.55	15.22	9.41	66.04 ± 5.6	0.09
Basal stems	0.74	1.74	1.14	3.62 ± 0.7	0.38	1.94	2.94	9.65	8.04	9.75	5.29	37.62 ± 9.2	0.10
Peelings (stump)	2.24	4.70	3.11	10.05 ± 0.7	1.39	6.76	6.77	26.04	21.13	23.17	15.95	99.83 ± 6.3	0.10
Peelings (stems)	1.29	3.22	2.12	6.63 ± 1.3	1.99	3.07	5.30	11.65	14.99	10.98	3.35	49.34 ± 13.5	0.23
Two days													
Roots (distal)	4.06	5.78	8.08	17.92 ± 2.0	1.61	10.69	6.29	21.72	16.38	21.96	14.73	91.77 ± 2.6	0.20
Roots (proximal)	4.88	7.04	8.70	20.62 ± 0.9	1.31	11.40	7.35	23.46	18.12	23.46	16.18	99.98 ± 7.6	0.21
Stump	3.56	5.90	6.82	16.28 ± 0.5	1.12	8.25	5.75	14.95	14.08	16.80	10.09	69.91 ± 4.2	0.23
Basal stems	1.14	2.50	3.35	6.99 ± 0.6	0.66	1.66	2.36	3.99	4.56	5.23	2.55	20.34 ± 3.1	0.34
Peelings (stump)	5.68	8.14	9.75	23.57 ± 2.0	1.99	9.69	9.09	17.22	19.50	20.35	14.80	90.65 ± 8.0	0.26
Peelings (stems)	3.95	6.81	8.96	19.72 ± 1.5	1.99	5.33	7.55	10.01	13.39	13.30	9.91	59.51 ± 4.6	0.33
Isa													
Roots (distal)	3.01	3.34	4.26	10.61 ± 0.5	1.22	8.03	6.89	13.71	13.26	15.23	8.79	65.90 ± 2.1	0.16
Roots (proximal)	2.41	4.11	5.57	12.10 ± 0.8	1.31	8.55	8.16	14.23	14.87	16.41	9.41	71.64 ± 3.8	0.17
Stump	2.84	3.92	3.65	10.41 ± 0.5	0.73	6.09	8.27	10.19	11.59	12.40	5.69	54.24 ± 3.1	0.19
Basal stems	2.32	2.66	2.87	7.85 ± 0.1	0.52	3.76	1.90	1.92	2.69	3.07	1.20	14.54 ± 0.9	0.54
Peelings (stump)	3.94	4.47	8.13	16.54 ± 0.5	1.21	8.73	12.08	14.18	18.62	17.71	10.03	81.34 ± 2.7	0.20
Peelings (stems)	2.06	4.01	5.83	11.90 ± 0.7	1.99	2.19	6.24	5.08	8.58	7.87	3.47	33.43 ± 2.0	0.36
Wichmannii													
Roots (distal)	3.22	3.21	7.69	14.12 ± 1.3	1.99	3.91	6.80	4.70	10.40	12.66	16.93	55.39 ± 9.2	0.25
Roots (proximal)	3.48	4.30	6.67	14.45 ± 1.7	1.84	4.39	3.29	6.53	5.52	16.22	17.02	52.97 ± 8.9	0.27
Stump	3.06	4.43	9.40	13.89 ± 0.1	1.45	3.49	9.65	3.69	3.13	11.90	12.96	44.82 ± 4.4	0.31
Basal stems	0.79	1.48	2.41	4.68 ± 0.5	0.91	0.57	3.53	0.96	1.15	3.78	4.22	14.21 ± 3.1	0.33
Peelings (stump)	3.12	1.39	5.18	11.69 ± 0.6	1.99	2.32	6.97	4.54	2.37	8.50	11.59	36.30 ± 4.2	0.32
Peelings (stems)	2.27	3.09	4.30	9.66 ± 0.8	1.99	1.06	5.12	3.11	2.46	6.30	7.65	25.71 ± 3.5	0.38

DMY, desmethoxy yangonin; *DHK*, dihydrokavain; *Y*, yangonin; *K*, kavain; *DHM*, dihydromethysticin; *M*, methysticin; *FKA*, flavokawain A; *FKB*, flavokawain B; *FKC*, flavokawain C

presented the highest M content with respectively 10.69, 11.40 and 8.25 mg/g in distal and proximal roots and stump. M is the only kavalactone which has been suspected of being potentially hepatotoxic (Li et al. 2011) although there is a need

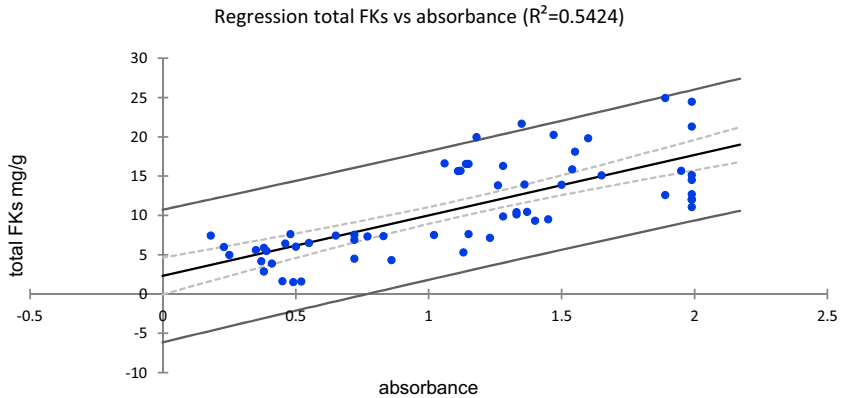


Fig. 4 Linear regression between total flavokawains content (FKs) and absorbance of acetonic extracts (measured at 440 nm) in five different plant parts (distal roots, proximal roots, stump, stump peelings, basal stems) of four different varieties ($n = 60$)

for more research. The total KLs of noble and two-day kava varieties (> 90 mg/g in the roots and > 60 mg/g in the stumps) were equivalent and were much higher than in isa and wichmannii plants. Total KLs were progressively decreasing from roots to stumps and stems in all four varieties. They remained high in the stump and stem peelings of all varieties and especially in the noble variety (99.83 and 49.34 mg/g) which is the most consumed and traded. This probably explains why these residues of consumption are sometimes used by unscrupulous traders for the adulteration of commercial powders (Duve and Prasad 1981). Our results are congruent with those of Smith et al. (1984) measured with HPLC.

The FK/KL ratio gives a fair indication of the composition of the plant part used to prepare the beverage. In the case of noble variety, these ratios were very low (0.06–0.09) for roots and stumps. In the case of two-day kava, these ratios are high (0.20–0.23) and were very high for non-consumable wild kava (0.25–0.31) with intermediate values for isa (0.16–0.19). For all four varieties, these ratios were much higher in the stumps and stems peelings. Based on these results, it appears that the FK/KL ratio could also be used as a criterion to characterise poor-quality kava products, the higher is this ratio and the poorer is the quality.

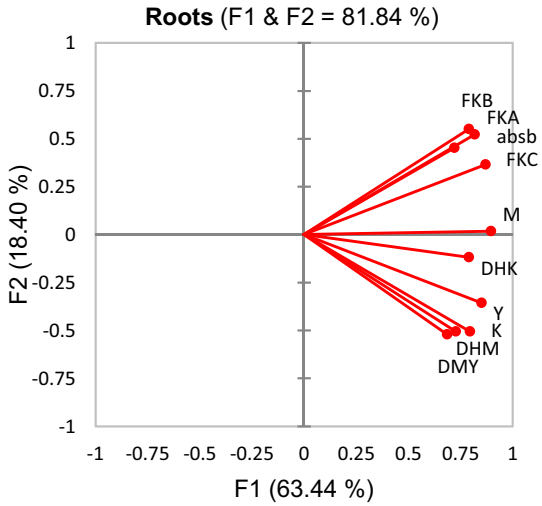
Flavokawains Variation in Exported Commercial Kava

Overall, 1053 samples collected on kava prepared for export trade and corresponding to 370 roots, 381 chips and 302 commercial powders were analysed using HPTLC and three FKs and six KLs were quantified. The results are presented in Table 3. The roots presented a significantly higher mean total FKs (11.07 mg/g) compared with chips (8.25 mg/g). The powders which often include basal stems and are sometimes suspected of being adulterated, presented lower FKs (7.79 mg/g). Kava is traded according to plant parts and roots are more expensive than chips and powders but the results of this study show that within each group of kava products, individual FKs and KLs presented significant variation with high coefficients of variation (CV) ranging from 20 to 50%. The variation within root samples was remarkable and ranged from one to ten, with a minimum of 2.53 mg/g to a maximum of 24.56 mg/g. The variation within chips ranged from 2.73 to

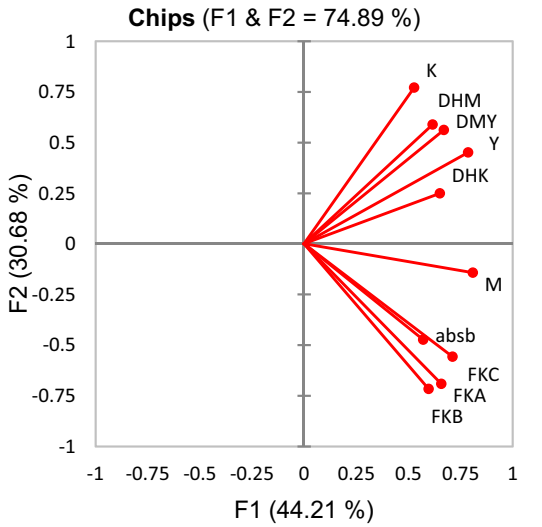
Table 3 Variation in flavokawain (FKs) and kavalactone (KLs) contents in roots, chips and powders exported from Vanuatu in 2017, 2018 and 2019. Values in mg/g correspond to peak areas converted into concentrations based on pure standards values

	Flavokawains				Absorbance	Kavalactones							FK/KL
	FKC	FKA	FKB	Total FKs		M	DHM	K	DHK	Y	DMY	Total KLs	
Roots													
n = 370													
Mean	2.70^{a}	4.09^{a}	4.28^{a}	11.07^{a}	0.86^{a}	9.67^{a}	8.24^{a}	24.09^{a}	14.81^{a}	20.88^{a}	15.56^{a}	93.25^{a}	0.12^{a}
Min	0.29	1.01	0.53	2.53	0.04	0.84	1.35	4.16	1.09	5.96	3.23	18.80	0.05
Max	6.58	9.09	9.53	24.56	1.69	17.83	25.53	33.97	23.69	29.88	22.21	137.34	0.29
SD	1.11	1.90	2.23	5.11	0.29	2.59	1.96	5.15	4.96	3.75	3.31	18.77	0.05
CV%	40.97	46.47	52.09	46.18	33.56	26.75	23.83	21.40	33.47	17.96	21.25	20.13	40.13
Chips													
n = 381													
Mean	1.84^{b}	3.31^{b}	3.09^{b}	8.25^{b}	0.56^{b}	6.15^{b}	6.48^{b}	17.12^{c}	11.16^{b}	15.25^{c}	10.24^{b}	66.42^{c}	0.13^{a}
Min	0.40	1.05	0.65	2.73	0.10	1.59	1.62	4.21	0.63	8.39	3.20	32.07	0.05
Max	5.11	7.64	7.35	18.03	1.50	11.97	29.56	28.68	20.96	27.10	20.47	123.16	0.29
SD	0.76	1.25	1.39	3.23	0.24	1.80	2.46	4.66	3.53	2.96	2.81	14.40	0.05
CV%	41.28	37.80	44.96	39.12	41.91	29.18	37.97	27.22	31.65	19.41	27.42	21.68	41.69
Powders													
n = 302													
Mean	2.05^{b}	3.04^{b}	2.69^{b}	7.79^{b}	0.68^{b}	7.18^{b}	7.61^{a}	20.07^{b}	11.27^{b}	17.42^{b}	11.63	75.17^{b}	0.11^{a}
Min	0.66	1.23	0.77	2.92	0.12	2.08	0.90	2.59	2.73	6.34	2.76	18.70	0.06
Max	5.17	7.05	6.61	16.41	1.50	13.68	24.05	32.71	24.15	28.03	20.94	131.35	0.32
SD	0.85	1.07	1.19	2.75	0.26	2.33	2.44	5.14	4.09	3.68	3.45	17.45	0.04
CV%	41.28	35.16	44.27	35.27	38.02	32.48	32.03	25.61	36.30	21.10	29.67	23.21	40.52

DMY, desmethoxy yangonin; *DHK*, dihydrokavain; *Y*, yangonin; *K*, kavain; *DHM*, dihydromethysticin; *M*, methysticin; *FKA*, flavokawain A; *FKB*, flavokawain B; *FKC*, flavokawain C; *SD*, standard deviation of the mean (computed using non-parametric tests: Kruskal-Wallis and Friedman). Means with different letters in superscript within each column are significantly different at $p \leq 0.05$



(a)



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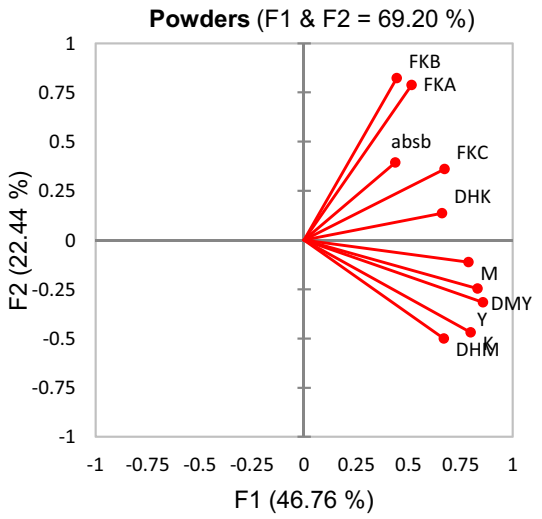

(c)

◀ **Fig. 5** Principal component analyses of roots (n = 370), chips (n = 381) and powders (n = 302) showing that the absorbance of acetonic extracts (absb) is correlated with the three flavokawain (FKA, FKB, FKC) contents for the three groups of kava products: roots (**a**), chips (**b**) and powders (**c**)

18.03 mg/g and in powders, from 2.92 to 16.41 mg/g. The variation in FK content has already been found to be very high in commercial kava sold in the USA, but only 25 samples were analysed with HPLC and UPLC (Martin et al. 2014). Our results confirm the great variation within each group: roots, chips and powders. The mean absorbance value of the acetonic extracts of the roots (0.86) was significantly higher than for the chips (0.56) with powders presenting an intermediate value (0.68). Principal components analysis of the three data sets (roots, chips and powders) are presented in Fig. 5 with the respective contributions of the variables. The results show that a great percentage of the variation is presented in axes 1 and 2: 81.84% for the roots, 74.89% for the chips and 69.2% for the powders.

As expected, the roots presented the highest KLs (93.25 mg/g) compared with powders (75.17 mg/g) and chips (66.42 mg/g). There is, however, tremendous variation for KLs depending on the age of the plant, the variety and environmental factors. For the three groups of products, the CVs were above 20%. An analysis of the variation between years of sample collection (2017, 2018 and 2019) revealed no significant means of differences (data not shown). Our HPTLC results are congruent with results obtained with HPLC (Bilia et al. 2004; Meissmer and Häberlein 2005; Martin et al. 2014; Liu et al. 2018) on a comparatively very limited number of samples. Nowadays, HPLC is used in importing countries to analyse the imported kava but the cost per sample is significant. HPTLC allows the fast and cost-efficient screening of hundreds of samples. In order to be efficient, routine chemical analysis of kava traded internationally has to be done before export from the Pacific.

Efficiency of the Colorimetric Test for Quality Control

A linear regression analysis conducted on all samples (n = 1053) revealed an R^2 value of 0.5211 indicating a positive relationship between the absorbance of the acetonic extract and the total FKs (Fig. 6). This colorimetric test cannot be used for the precise quantification of FKs but it represents a qualitative assessment of the total FK content. Except for M which is yellow, all KLs are white crystals when pure and FKs are orange. Correlation coefficients between the FKs and KLs are presented in Table 4. The individual compounds presenting the highest coefficients were FKA, FKB, FKC and M with, however, higher correlations in the roots than in the chips and powders. Other pigments were not observed on

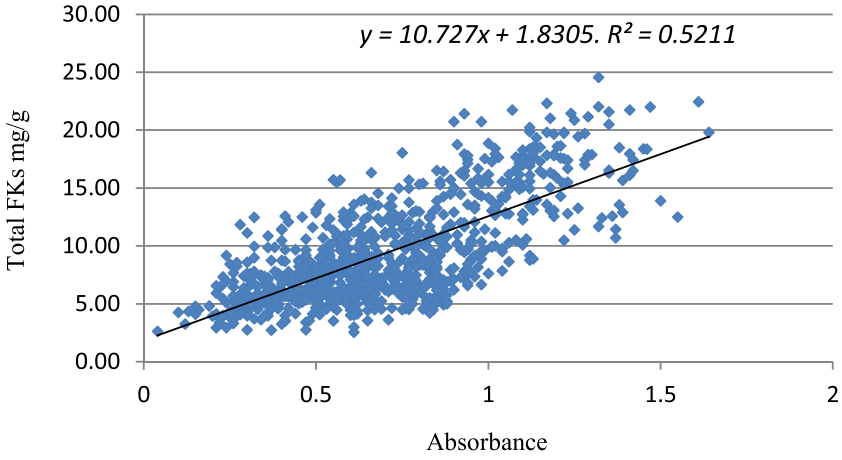

Fig. 6 Linear regression between total flavokawain content (FKs) and absorbance of acetonic extracts (measured at 440 nm) in 1053 samples of commercial kava (roots, chips, powders) exported from Vanuatu in 2017, 2018 and 2019

HPTLC plates and were therefore in minor quantity compared with FKs. Consequently, the colorimetric test can be considered as a fair assessment of the FK content in the acetonic extract. As acetone has been shown to be the most efficient solvent for KL and FK extraction (Xuan et al. 2008), the high absorbance of the extract is a good indication of the presence of non-KL compounds in the extract. As KLs are the molecules of interest for kava consumers, the highest is the absorbance, the lowest is the quality. At present, Vanuatu authorities control kava batches prior to export using the colorimetric test of the acetonic extract which has been shown to be suitable for differentiating noble, two-day and wichmannii varieties (Lebot and Legendre 2016). The upper limit above which the commercial batches are presently rejected is 0.9 of absorbance at 440 nm. In the present study, 207 samples (155 roots, 29 chips and 23 powders) had an absorbance above 0.9 which represents approximately 19.66% of the total (1053).

FKs are present in very low proportions in kava, the highest value detected in the present study, being 24.56 mg/g (~ 2.5%DW), but their mean content is only 11.07 mg/g (~ 1.1%DW) in the roots and less in the chips and powders. These molecules are non-soluble in water and are not known to produce a physiological effect, unlike KLs. The fact that they are present in very low contents in noble variety reveals that they do not participate in the beverage quality. On the other hand, they might represent a potential risk if kava is used to prepare nutraceutical or pharmaceutical products based on extracts obtained with solvents which can concentrate high levels of FKs, although this has not yet been demonstrated. Several studies have shown that they exhibit interesting anticancer properties and there is therefore a need for further research to assess their role in the plant and their presence in the beverage, if any.

Conclusions

FKs are mostly concentrated in the surface cell layers of the kava plant, the bark of the stump and stem, indicating that they may play a defensive and/or protective role for the plant. Chalcones are flavonoids and this family of molecules is often reported as protecting other plant species. FKs are in very low proportions in the noble variety used for daily drinking and appreciated for its pleasant relaxing effect. This may suggest that their contribution to the beverage quality is very limited, especially as FKs are not water soluble. Compared with HPLC or UPLC protocols, HPTLC allows the rapid and cost-efficient quantification of FKs. This analysis can be conducted in the Pacific before export to control the quality of internationally traded kava. In the present study, we have analysed 1053 kava samples and the results represent a useful database for further studies. The colorimetric test based on the absorbance of the acetonic extract is useful. Although it does not represent an accurate quantification of the FKs, it gives a fairly good indication of their content in the kava products.

Table 4 Spearman correlation coefficients between the absorbance of the acetonic extracts and the individual compounds

Variables	*n*	FKC	FKA	FKB	M	DHM	K	DHK	Y	DMY
Roots	370	0.731**	0.751**	0.746**	0.630**	0.280**	0.332**	0.446**	0.456**	0.340**
Chips	381	0.570**	0.601**	0.572**	0.521**	0.129 ns	− 0.036 ns	0.152**	0.198**	0.112 ns
Powders	302	0.414**	0.358**	0.364**	0.378**	0.062 ns	0.150**	0.222**	0.255**	0.190**

**Significant at $p < 0.01$ and $n > 300$, r value at 1% = 0.148

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Compliance with Ethical Standards

Conflict of Interest Vincent Lebot declares that he has no conflict of interest. Juliane Kaoh declares that she has no conflict of interest. Laurent Legendre declares that he has no conflict of interest.

Ethical Approval This article does not contain any studies with animals performed by any of the authors.

Informed Consent Informed consent is not applicable in this study.

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