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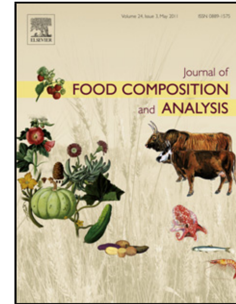

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Original Research Article

Colorimetric assessment of kava (*Piper methysticum* Forst) quality

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Highlights:

- Kava is a traditional and major cash crop in the South Pacific
- First characterization of diethyl ether root extracts for kava
- GC-LC/MS analyses and UV/Vis absorption spectra define three chemotypes
- Chemotypes correspond to known health beneficial and toxicity genetic classes
- Absorbance ratio 250/290 ascertains kava batch chemical suitability

ABSTRACT

The present study aimed at evaluating the potential of diethyl ether extracts UV/Visible (UV/VIS) absorbance for assessing the suitability of commercial lots of kava (*Piper methysticum*). The UV/VIS absorption spectra of diethyl ether root extracts of 15 cultivars clustered them into three groups in parallel to their known genetic relatedness and their chemical composition determined by GC-MS and LC-MS analyses. Absorption peaks at 250 nm and 290 nm respectively corresponded to kavain, the most health-promoting kavalactone, and dihydromethysticin a non-desirable kavalactone. The absorbance peak at 340–350 nm reflected the yellow coloration of the extract, which was mainly due to the undesirable flavokavins, desmethoxyyangonin and yangonin. Ratios of absorbance values at 250 nm and 290 nm significantly differentiated all three groups of cultivars, namely 'noble' which provide health benefits from 'two-day' and 'wichmannii' that are health damaging. These results provide a robust and rapid colorimetric test for routine control of a critical aspect of the quality of kava batches.

Keywords: chemotype; kava; flavokavain; food analysis; food composition; food quality assessment; kavalactone; *Piper methysticum*.

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1. Introduction

Kava (*Piper methysticum* Forst.), a member of the piper family Piperaceae, is a small shrub that is endemic to South Pacific islands and Papua New Guinea. Cold water extraction of its underground organs generates the traditional beverage of Pacific island countries used for ceremonial and recreational purposes (Lebot *et al*., 1997). Over the past century, kava beverage has become popular worldwide and now represents one of the major cash crops in Fiji, Pohnpei, Samoa, Tonga, and Vanuatu. Upon ingestion, it promotes relaxation and a sense of well-being without compromising cognitive capacities and triggering addictive behaviors (Sarris *et al*., 2011). It is also claimed to bear medicinal properties, such as attenuation of menopausal symptoms, neuroprotection, anti-bacterial, anti-mycotic, anti-epileptic, spasmolytic, analgesic, locally anesthetic diuretic and soporific (Showman *et al*., 2015) and crop protective effects (Xuan *et al*., 2003). In sharp contrast, several cases of hepatotoxicity have been reported and prompted European authorities to temporarily ban kava imports (BfArM, 2003; Bilia *et al*., 2002; Clough *et al*., 2003). Reasons for these health problems stem from increased demand, which may have prompted traders to sell extracts from non-traditionally-used and inappropriate plant parts or varieties. Stem peelings and leaves, indeed, specifically accumulate pipermethysticin, a toxic alkaloid (Dragull *et al*., 2003; Jhoo *et al*., 2006).

The species *P. methysticum* also appeared to include three genetically and chemically distinct groups (Lebot *et al*., 1991; VandenBroucke *et al*., 2015). The most ancient one called 'wichmannii' comprises wild ancestors which are currently only found in Papua New Guinea, the Solomon Islands and Vanuatu and from which a group of cultivars collectively called 'two-day' was derived. This latter group of cultivars served as a reservoir for the selection of a third group of cultivars called 'noble' which are currently only multiplied vegetatively because of their sterility. Diversification among the

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'noble' cultivars arose *via* the section of somaclonal variants (Lebot & Lévesque, 1989). According to local tradition, only these later cultivars are suitable for daily consumption. Though they may differ slightly in their phytochemical content because of genetic, ontogenic and cultivation differences (Wang *et al*., 2013; WHO, 2007), they exhibit great chemical differences from two-day and wichmannii cultivars by having a much higher proportion of kavalactone (KL) kavain (K) and a near complete absence of a group of chalcones called flavokavains (FKs) (Figure 1) (Lebot *et al*., 2014). This agrees with the suspected hepatotoxicity of FKs, and especially FKB, the major FK (DiSilvestro *et al*., 2007, Zhou *et al*., 2010). Though all three groups of kava cultivars can be distinguished by the morphology of their aerial parts, the slighter differences between two-day and noble cultivars only supports a separation of the species into two varieties, *P. methysticum* var. *methysticum* (noble and two-day) and *P. methysticum* var. *wichmannii* (Applequist & Lebot, 2006). This, and the fact that traded plant material consists of powdered, dry underground organs, make attribution of commercially sold material to a specific cultivar, or even a group of cultivars, impossible. This uncertainty and misidentification may have been the cause of the reported liver problems (Kuchta *et al*., 2015; Martin *et al*., 2014; Teschke *et al*., 2011).

In an effort to support their kava industry, Pacific island countries are aiming to set up quality standards (FAO/WHO, 2013; IKEC, 2004; SPC, 2001; Teshcke et al., 2011; Vanuatu Legislation, 2002). A variety of sturdy and reliable HPLC- and GC-based protocols have been developed (Bilia *et al*., 2004; Ganzera & Khan, 1999; Gaub *et al*., 2004a) for the quantitative analysis of the six major KLs (methysticin M, dihydromethysticin DHM, kavain K, 7,8-dihydrokavain DHK, desmethoxyyangonin DMY, yangonin Y) and the two major FKs (FKA, FKB) (Figure 1). Detection is made through diode array and mass spectrometer detectors because these substances display characteristic UV/Visible (UV/Vis) absorption spectra and masses (Meissmer & Häberlein, 2005). The nature of the extraction solvent has some importance, as the highly non-polar solvent, hexane, and the very polar solvent, water, both have lower extraction capacities in agreement with the medium polarity of KLs and FKs. A comparison of the medium polarity solvents methanol, ethanol, dichloromethane, chloroform and

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acetone revealed only minor differences in extraction capacity (Xuan *et al*., 2008). The recent development of HP-TLC (Lebot & Legendre, 2014) and NIRS-based analytical protocols (Gaub *et al*., 2004b; Gautz *et al*., 2006; Wang *et al*., 2010) have respectively reduced solvent volumes and analytical time while still being able to distinguish the three major kava chemical groups. They nevertheless require equipment that is too costly for most of the Pacific island countries that produce kava. These methods are also too time-consuming for surprise inspections by control officers. For this reason, direct UV absorbance measurements have been applied to acetone extracts of kava root powder (Lebot & Legendre, 2016). Through the analysis of more than 200 plants, a positive correlation was found between the absorbance at 400 nm and total FKs content, in agreement with the fact that FKs and the KLs Y and DMY are yellow. Ratios of K/total KLs and total FKs/KLs also correlate with absorbance at 400 nm and allow some degree of discrimination of kava cultivars according to their genetic (i.e. toxicity) group.

Nevertheless, this approach suffers from several shortcomings. A multivariate principal component analysis of the 308 samples using these characteristics revealed a wide dispersion of data points with some overlap of the 95% level distribution ellipses of the three genetic groups, therefore suggesting a significant risk of mistaken assignment of unknown samples to a chemical class. Absorbance values alone were also only capable of distinguishing wichmannii cultivars from the others, the separation of two-day and noble cultivars additionally requiring the use of substance content ratios obtained by HP-TLC. The problem potentially stems from the high absorbance of acetone below 340 nm, a value that is greater than the absorbance maxima of most KLs and FKs. The present study therefore aimed at evaluating the potential of diethyl ether for extraction, as an alternative to acetone for the development of a sturdier UV/VIS absorbance-based assay that can ascertain the suitability of commercial lots of kava root powder for human consumption.

2. Materials and methods

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2.1. Plant materials

All plants were grown at the Vanuatu Agricultural Research and Technical Center (VARTC) on Santo Island (Vanuatu) (80 m a.s.l., 15º26'7'' S, 167º11'5'' E), at 2.8 km from the seashore, on a deep and fertile brown soil, covering a limestone plateau. Underground organs were harvested when plants were mature, after three years of growth. Roots of noble (*Kelai, Borogu, Palarasul, Ni Kawa Pia, Sese, Silese, Pia*), two-day (*Twoday, Palisi, Sentender*) and wichmannii (*Wichmannii, SK155, Mele Liap, Sini Bo, Malogu Buara*) varieties were harvested, washed and cut into small cubic pieces of approximately 2 x 2 cm before being oven-dried at 60–80 °C until weight was constant. They were reduced to powder with a benchtop high speed bead homogenizer (FastPrep®-24; MP Biomedicals, Santa Ana, CA).

2.2. Comparative extraction of kava root by different solvents

Dry kava root powder (300 mg) was mixed with 3 mL of solvent (water, acetone, chloroform, methanol, ethanol, hexane or diethyl ether) and sonicated for 15 min at room temperature before being centrifuged at 3 000 *g* for 5 min. Supernatants (70 µL except for the water extracts for which only 30 µL were used) were hand-spotted and chromatographed on an RP-18 F254 thin-layer chromatography (TLC) plate with aluminum support (Macherey-Nagel, Neumann, Germany). Elution was made in a closed chamber with dichloromethane as elution solvent. Substances were visualized with white light and under illumination at 254 nm and 366 nm. In parallel, aliquots of crude extracts were subjected to GC-MS and LC-MS analyses.

To assess the completeness of the extraction of yellow substances by diethyl ether, the above pellets of diethyl ether extracted kava root powder were first subjected to a similar extraction with acetone and this second extract was also chromatographed by TLC as described above. Alternatively, the pellets were subjected to a second extraction with diethyl ether and the resulting

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supernatant subjected to comparative quantitative analysis of major KLs and FKs by GC-MS and LC-MS in parallel to crude diethyl ether extracts.

To determine the nature of the two yellow substances of the diethyl ether extracts that migrated close to the solvent front on the above TLC system (R_f = 0.94 and R_f = 0.96), the yellow bands were scraped off from the TLC plates and the detached silica was extracted with methanol for 10 min. Eluates were then subjected to GC-MS and LC-MS analyses.

2.3. Determination of UV/VIS absorbance

Dry kava root powder (100 mg) was mixed with diethyl ether (4 mL) and sonicated for 15 min at room temperature before being centrifuged at 3 000 *g* for 5 min. The supernatant was collected and the pellet re-extracted similarly. Both supernatants were combined, diluted 100 fold with diethyl ether and their UV/Vis absorbance recorded from 210 to 500 nm with a UV/Vis spectrophotometer (Agilent 8453; Agilent, Santa Clara, CA) using diethyl ether for blank correction. Absorbance values obtained every 10 nm from 210 nm to 420 nm were used for statistical analyses.

2.4. GC-MS analyses

Diethyl ether or hexane extracts were analyzed by GC-MS on an Agilent 7890A GC (Agilent, Santa Clara, CA) instrument coupled to an electron impact ionization chamber and a quadrupole-based mass analyzer (Agilent 7000A, Santa Clara, USA). An aliquot of 1 µL was injected in splitless mode on a DB5-MS fused silica capillary column (60 m x 0.25 mm, 0.25 µm film thickness) with helium carrier gas (2.25 mL min^{-1}). Injector temperature was 290 °C and initial column temperature was 70 °C. The column was held at this temperature for 3 min, then heated to 300 °C at 4°C min^{-1} and finally kept at 300°C for 5 min. MS ionization voltage was set at 70 eV, scan rate at 500 amu s^{-1}, quadrupole temperature at 150 °C and ion source temperature at 230 °C. MS scan range was *m/z* 35–500. Peaks were automatically integrated and baselines were corrected manually if needed. Less

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than 5% total ion current (TIC) peak area variations were observed between replicate injections. Contaminants originating from the extraction solvent or the column were identified (and disregarded from later analyses) by comparison with control injections of diethyl ether or hexane. Substances were annotated on the basis of their retention times and fragmentation mass spectra. Retention times of a series of *n*-alkanes (C8–C40) (Sigma-Aldrich, St Quentin Fallavier, France) were used to convert retention times into Kovats' retention indices (van den Dool & Kratz, 1963). Major KLs and FKs were identified by comparison with pure standards respectively purchased from Sigma-Aldrich (Fluka, St Quentin Fallavier, France) and LKT Laboratories (St Paul, MN).

2.5. LC-MS-MS analyses

For LC-MS analysis, diethyl ether extracts were dried under speed-vacuum and re-suspended in methanol at 1 mg/mL. An aliquot of 0.5 µL was injected onto a C-18 UHPLC column (Poroshell 120 EC-C18, 3.0 x 100 mm 2.7 µm, Agilent) maintained at 60 °C and powered by an Agilent 1290 UHPLC connected to an Agilent 6530 DAD-ESI-Q-ToF in positive and negative modes. Elution was made at 1 mL/min with 65% solvent **A** (0.4% formic acid in water) and 35% solvent **B** (acetonitrile) for 7 min followed by a 10 min linear gradient up to 100% solvent **B**, which was maintained for 3 min. It was brought back to 65% solvent **A** in 0.5 min and re-equilibrated at these conditions for 3.5 min. The ESI source was optimized as follows: scan spectra from *m/z* 100–2000, capillary voltage 3.5 kV, fragmenter at 120 V, fixed collision-induced dissociation (CID) energy at 20 eV. Nitrogen was used as the nebulizing gas with a flow rate of 11 mL/min and a temperature of 310 °C at 40 psi. Ionization was more efficient under positive mode so that this mode was used for later data analyses. The DAD module was set at 280 nm and 360 nm. It recorded spectra from 190 to 900 nm. UV/Vis spectra and HRMS characteristics were extracted using MassHunter Quantitative Analysis software (Agilent Technologies, Santa Clara, USA) by using default settings.

Ten µL of each analyzed sample were mixed and this quality control (QC) sample was chromatographed every 10 injections to correct for potential drifts in ionization efficiency and/or

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retention time. The analysis of this QC sample was also used to select substances to quantify in each sample. For this, every peak of the TIC chromatogram obtained under positive ionization was integrated. Peaks had their mass spectra analyzed to select all masses that had a sodium adduct with a similar retention time. Substances were therefore annotated with their high resolution mass and retention time. Major KLs and FKs were identified by comparison with pure standards. Extracted ion chromatograms (EIC) corresponding to pseudomolecular ions of all these substances were then integrated manually in kava extract chromatograms using Mass Hunter software (Agilent, Santa Clara, CA) and the peak areas used for statistical analyses.

2.6. Statistical analyses

All statistical analyses were conducted with the open source software R (R Core Team, 2014) using ade4 and RVAideMemoire packages. Individual tests are indicated in the text.

3. Results and discussion

3.1. Extraction of colored pigments

With the aim of finding a solvent that selectively extracted known pigments from kava roots, dry kava root powders of one noble (*Borogu*) cultivar and one wichmannii (*Wichmannii*) cultivar were sonicated with either water, acetone, chloroform, methanol, ethanol, hexane or diethyl ether. As shown in Figure 2, all extracts of the wichmannii cultivar were more intensely pigmented (yellow to brown) than their noble cultivar counterparts, in agreement with previous observations using acetone extracts (Lebot *et al*., 2014, Lebot *et al*., 2016). This confirms that wichmannii cultivars tend to accumulate larger amounts of the naturally yellow FKs (Lebot *et al*., 2014). Still in agreement with the greater ability of chloroform and acetone to extract the colored FKs (Xuan *et al*., 2008), extracts with these solvents were the darkest for the wichmannii cultivar. However, this was not the case with the noble cultivar for which water extracts were the brownest (Figure 2A). Because water is a

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poor FK solubilizing solvent and because noble cultivars are expected to contain little of these substances, water extracts probably contain additional colored substances besides the colored KLs, Y or DMY. This is confirmed by a comparative TLC analysis of these extracts (Figure 3). Although not ideal for individual KL analysis, the solvent system that was used had the advantage of sorting out yellow pigments that migrated at high R_f (0.94–0.96) at the level of FKA/B, from some that smeared amongst major KLs ($0.1 < R_f < 0.8$) and brown substances that did not migrate.

All extracts except the aqueous and hexane extracts generated 2 yellow bands of similar coloration at high R_f (these were less intense in hexane extracts and barely visible in aqueous extracts). The 2 bands were scraped off together from the TLC plates, the silica extracted with methanol and the eluate analyzed by GC-MS and LC-MS. It only contained two substances that were identified as being FKA and FKB, by comparison of their mass spectra and retention times with standards. As expected, they were more abundant in wichmannii extracts. All extracts also yielded a faint and smeared yellow coloration where KLs migrate. This coloration was weakest in the water and hexane extracts while being equivalent in all other extracts, in agreement with the lesser ability of these two earlier-used solvents to extract KLs. However, most of the brown coloration of the extracts was due to substances that did not migrate in this chromatographic system. Efforts to have them migrate as distinct spots have remained unsuccessful, and their nature could not be determined. They are, however, very polar and extracted readily in water. They accounted for a greater proportion of the coloration of the water, acetone, chloroform, methanol and ethanol extracts of the noble cultivar than of the wichmannii cultivar. Hexane extracted the smallest quantity of these brown substances so that the faint yellow coloration of these extracts was nearly entirely due to FKA and FKB. Some hexane-extracted substances, however, precipitated over time (*circa* 1 h), which hampered efforts to analyze them by spectrophotometry. Hexane was also less capable of extracting FKA and FKB. A GC-MS quantification of these two substances indicated that hexane extracted, respectively on average, 28% and 33% of the quantities present in diethyl ether extracts. Diethyl

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ether extracts were therefore selected as a more efficient and selective extraction solvent for yellow FKs and KLs.

In order to determine whether diethyl ether completely extracted yellow FKs and KLs, two successive extractions were made on noble (*Borogu*) and wichmannii (*wichmannii*) cultivar. The second extractions yielded extracts with absorbances at 348 nm that dropped by 83% and 81% respectively (Standard deviation of 0.7% and 1.5%; *n* = 3). They also extracted little additional material of the non-migrating brown substances (data not shown). If acetone was used as second extraction solvent, extraction of FKs was not improved (Figure 3) but acetone contained more brown, non-migrating substances. A double extraction with diethyl ether was therefore considered sufficient to quantitatively and more specifically extract yellow FKs and KLs.

3.2. Chemical composition of diethyl ether extracts

In order to further improve our knowledge of the chemical composition of diethyl ether extracts, extracts of seven noble, three two-day and five wichmannii cultivars were first subjected to GC-MS analysis according to previously validated protocols (Xuan *et al.*, 2008). Examples of chromatograms can be found in the supplementary material. A total of 58 substances were annotated on the basis of their Kovats retention index and mass spectrum. They made up most of the trace area of the chromatograms. Among them, five of the six major KLs, FKA and FKB were identified by comparison with pure standards (Table 1). Only methysticin (M) was not detected in agreement with the known thermal instability of this substance in the injection port (Bilia *et al.*, 2004). A principal component analysis (PCA) conducted with the peak areas of the 58 annotated substances clearly separated the kava samples according to their toxicity class (Figure 4A). Noble and wichmannii cultivars opposed each other on the first axis while two-day cultivars had an intermediate position on the first axis and differed on the second axis. A similar plot was also obtained when the analysis was only conducted with the peak areas of the identified KLs and FKs (data not shown). The position of the chemical vectors on this later PCA plot suggested that noble

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cultivars were characterized by higher levels of kavain while, on the other hand, wichmannii cultivars contained higher levels of dihydromethysticin, desmethoxyyangonin and FKB. This, and the intermediate positioning of the two-day cultivars on the PCA plots agree with the proposed two-step selection of kava cultivars (Lebot & Lévesque, 1989) and previously published chemotypes for these three groups of cultivars (Lebot & Lévesque, 1996; VandenBroucke *et al*. 2015).

Because many of the GC-MS peaks may result from the thermal degradation of KLs, the above samples were also analyzed by LC-MS. Examples of chromatograms can be found in the supplementary material. The analysis of seven noble, three two-day and four wichmannii cultivars allowed the annotation of 22 substances (Table 2) on the basis of their high precision mass and retention time. These substances were selected because they were the only ones for which their mass fragment under positive ionization ($[M+H]^+$) was accompanied by the one of a sodium adduct ($M+Na]^+$) at a similar retention time. All six major KLs and the two major FKs were among these substances and were identified by comparison with standards. Based on their UV/VIS absorption spectra, compounds (**9**), (**10**), (**12**), (**13**) were suggested to be flavanones in agreement with the previous description of this class of substances in kava root extracts (Wu *et al*., 2002; Xuan *et al*., 2008). A multivariate PCA conducted with the peak areas of extracted ion chromatograms of the 22 annotated substances revealed an excellent separation of the three groups of cultivars (Figure 4B). It confirmed earlier reports using acetone extracts (Lebot *et al*., 2014; Wang *et al*., 2013) that the two-step sequential selection of kava cultivar groups resulted in a stepwise increase in kavain content and a stepwise decrease in dihydromethysiticin (Figure 4C – pairwise Wilcoxon test with significance at $p < 0.05$). Noble cultivars were also characterized by low FKs content. Two-day cultivars resembled noble cultivars in their Y and DMY contents but differed from other groups in having higher FKs and M contents. DHK content was found to be at a similar level in all three groups. Therefore, the greater yellow coloration of wichmannii cultivar extracts was due to higher levels of DMY, FKA and FKB and that of two-day cultivars to Y, FKA and FKB. The fainter yellow color of noble cultivars was due to small quantities of these substances and to a larger quantity of Y. Compounds (**14**) and (**15**) may also

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be yellow, based on their maximum absorption wavelengths. However, their very small contribution to the overall trace area at 355 nm suggests that their contribution to the overall coloration of the extracts is, at best, minimal.

In agreement with the proposed position of wichmannii cultivars as wild ancestors of the two other selected groups (VandenBroucke *et al*., 2015), wichmannii samples spread most on the PCA plot (Figure 4B). This large dispersion was mostly linked to the contents of compounds (**12**), (**13**), (**16**), (**18**), (**19**) and (**20**). Compounds (**3**), (**9**), (**11**) were variable among all samples while, among unidentified substances, compounds (**10**), (**14**), (**15**) and (**17**) were more abundant in wichmannii cultivars and compound (**6**) in noble cultivars.

3.3. Absorbance of diethyl ether extracts as a tool to assign kava cultivars to chemical classes

Because major KLs and FKs display different UV-absorption maxima (Lebot *et al*., 2014), an evaluation was made of the UV/Vis absorption spectra of crude diethyl ether extracts of kava roots. Visual inspection of these spectra clearly grouped them according to kava genetic classes (Figure 5). Noble cultivar extracts exhibited maxima at 250 nm and 350 nm, two-day cultivar extracts at 260 nm, 290 nm and 350 nm and wichmannii cultivar extracts at 260 nm, 285 nm and 340 nm. Based on the known absorption maxima of major KLs and FKs, the bathochromic shift of absorbance from 340 nm to 350 nm was most likely due to the lesser abundance of DMY in noble and two-day cultivars. The gradual decrease in intensity of the absorption peak at 250 nm from noble to two-day to wichmannii cultivars parallels the decreases in K content observed during GC-MS and LC-MS analyses and agrees with the absorption maximum of K around 250 nm. Similarly, the stepwise increase of DHM among these three groups of cultivars is reflected by an increase of absorbance at 290 nm, close to an absorption maximum of DHM. A PCA plot of the absorbance values of kava cultivar extracts taken every 10 nm from 210 nm to 420 nm separated kava cultivars according to their genetic class (data not shown). This was mostly due to the fact that accumulated contents of KLs and FKs were highest for wichmannii cultivars and lowest for noble cultivars (respectively highest and lowest absorptions

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at all wavelengths). To make spectra more comparable (less subject to cumulated quantities of substances as opposed to relative quantities), absorption values were normalized to the one at 350 nm because all cultivar extracts had an absorption maximum at, or near, this value. This improved the separation of cultivars according to their genetic class in a PCA plot as shown in Figure 6 and showed that wichmannii cultivar extracts were characterized by higher relative absorption at 290–340 nm and those of noble cultivars by higher relative absorptions at 240–260 nm. Two-day cultivars had an intermediate position and differed from the other groups by their relative absorption at 280–300 nm.

Because absorbance peaks at 250 nm and 290 nm reflect abundances of two KLs which differentiate all three groups of cultivars, ratios of absorbance values at these two wavelengths were used to differentiate kava cultivars according to their genetic class (Figure 7). Differences were significant at $p = 0.004$ with a non-parametric Kruskal-Wallis test. Pairwise Wilcoxon rank sum tests further confirmed that all three means are different from each other at $p \leq 0.05$.

4. Conclusion

The value of the ratio of absorbances at 250 nm and 290 nm of diethyl ether kava root powder extracts differentiate kava cultivars according to their genetic (i.e. chemotypic and toxicity) class. Not only is this distinction statistically significant with the 15 cultivars used in this study, but it is supported by the fact that these absorbances reflect, respectively, quantities of kavain and DHM, two substances known to be associated with kava beverage benefit/toxicity and to exhibit opposite stepwise content changes from noble to two-day and to wichmannii cultivars. This absorbance ratio more reliably distinguished cultivar classes than the previously suggested measure of absorbance at 400 nm (Lebot & Legendre, 2016), which was also unable to distinguish two-day and noble cultivars. Based on the results of this study, one of the reasons is that acetone absorption masks most of the absorption from major KLs and FKs, so that the absorption of acetone extracts mostly reflects the total quantity of KLs and FKs rather than respective individual quantities, a parameter that only

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remotely characterized kava classes. In addition, acetone extracts a large quantity of unknown brown substances that are abundant in noble cultivars, absorb at 400 nm and present no guarantee of differential accumulation in kava roots according to the cultivar class.

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Appendix A. Supplementary material

Supplementary material associated with this article can be found in the online version.

References

Applequist, W.L., & Lebot, V. (2006). Validation of *Piper methysticum* var. *wichmannii* (Piperaceae). *Novon, 16*, 3-4.

BfArM (2003). http://www.bfarm.de/de_ver/arzneimittel/amirisiken/stufenplan/Besch-Kava-Final.pdf. accessed 10.27.16.

Bilia, A.R., Gallori, S., Vincieri, F.F. (2002). Kava-kava and anxiety: growing knowledge about the efficacy and safety. *Life Science, 70*, 2581-2597.

Bilia, A.R., Scalise, L., Bergonzi, M.C., Vincieri, F.F. (2004). Analysis of kavalactones from *Piper methysticum* (kava-kava). *Journal of Chromatography B, 812*, 203-214.

Clough, A.R., Bailie, R.S., Currie, B. (2003). Liver function test abnormalities in users of aqueous kava extracts. *Journal of Toxicology. Clinical Toxicology, 41*, 821-829.

DiSilvestro, R.A., Zhang, W., DiSilvestro, D.J. (2007). Kava feeding in rats does not cause liver injury nor enhance galactosamine-induced hepatitis. *Food and Chemical Toxicology, 45*, 1293-1300.

ACCEPTED MANUSCRIPT

Dragull, K., Yoshida, W.Y., Tang, C.S. (2003). Piperidine alkaloids from *Piper methysticum*. *Phytochemistry, 63*, 193-198.

FAO/WHO (2013). Report of the 12th Session of the FAO/WHO Coordinating Committee for North America and the South West Pacific. In Codex Alimentarius Commission, *Joint FAO/WHO Food Standard Programme* (pp. 1-26). REP13/NASWP, Rome.

Ganzera, M., & Khan, I.A. (1999). Analytical techniques for the determination of lactones in *Piper methysticum* Forst. *Chromatographia, 50*, 649-653.

Gaub, M., von Brocke, A., Roos, G., Kovar, K.A. (2004a). High performance liquid chromatography ion spray-mass spectrometry (HPLC/CIS/MS): A new tool for the analysis of non polar compound classes in plant extracts using the example of *Piper methysticum*. *Phytochemical Analysis, 15*, 300-305.

Gaub, M., Roeseler, C., Roos, G., Kovar, K.A. (2004b). Analysis of plant extracts by NIRS: simultaneous determination of kavapyrones and water in dry extracts of *Piper methysticum* Forst. *Journal of Pharmaceutical and Biomedical Analysis, 36*, 859-864.

Gautz, L., Kaufusi, P., Jackson, M.C., Bittenbender, H.C., Tang, C.S. (2006). Determination of kavalactones in dried kava (*Piper methysticum*) powder using near-infrared reflectance spectroscopy and partial least-squares regressions. *Journal of Agricultural and Food Chemistry, 54*, 6147-6152.

IKEC (2004). Recommendations of the International Kava Conference. Suva, Fiji.

Jhoo, J.W., Freeman, J.P., Heinze, T.M., Moody, J.D., Schnackenberg, L.K., Beger, R.D., Dragull, K., Tang, C.S., Ang, C.Y.W. (2006). *In vitro* cytotoxicity of nonpolar constituents from different parts of kava plant (*Piper methysticum*). *Journal of Agricultural and Food Chemistry, 54*, 3157-3162.

Kuchta, K., Schmidt, M., Nahrstedt, A. (2015). German kava ban lifted by court: the alleged hepatotoxicity of kava (*Piper methysticum*) as a case of ill-defined herbal drug identity, lacking quality control, and misguided regulatory politics. *Planta Medica, 81*, 1647-1653.

ACCEPTED MANUSCRIPT

Lebot, V., & Legendre, L. (2016). Comparison of kava (*Piper methysticum* Forst.) varieties by UV absorbance of acetonic extracts and high-performance thin-layer chromatography. *Journal of Food Composition and Analysis, 48*, 25–33.

Lebot, V., & Lévesque, J. (1989). The origin and distribution of kava (*Piper methysticum* Forst. f. Piperaceae): a phytochemical approach. *Allertonia, 5*, 223-380.

Lebot, V., & Lévesque, J. (1996). Genetic control of kavalactone chemotypes in *Piper methysticum* cultivars. *Phytochemistry, 43*, 397-403.

Lebot, V., Arhadya, M., Manshardt, R. (1991). Geographical survey of genetic variation in kava (*Piper methysticum*). *Pacific Science, 45*, 169-185.

Lebot, V., Do, T.K., Legendre, L. (2014). Detection of Flavokavins (A, B, C) in cultivars of kava (*Piper methysticum*) using HP-TLC. *Food Chemistry, 151*, 554-560.

Lebot, V., Merlin, M., Lindstrom, L. (1997). Kava: The Pacific Elixir. Healing Arts Press, Rochester, Vermont.

Martin, A.C., Johnston, E., Xing, C., Hegeman, A.D. (2014). Measuring the chemical and cytotoxic variability of commercially available kava (*Piper methysticum* G. Forster). *PlosOne, 9*, 11/ee111572.

Meissmer, O., & Häberlein, H. (2005). HPLC analysis of flavokavins and kavapyrones from *Piper methysticum* Forst. *Journal of Chromatography B, 826*, 46-49.

R Core Team (2014). R: A language and environment for statistical computing. R Foundation for Statistical Computing. URL http://www.R-project.org/. Vienna, Austria (accessed 10.24.16).

Sarris, J., LaPorte, E., Schweitzer, I. (2011). Kava: a comprehensive review of efficacy, safety, and psychopharmacology. *Australian and New Zealand Journal of Psychiatry, 45*, 27–35.

Showman, A.F., Baker, J.D., Linares, C., Naeole, C.K., Borris, R., Johnston, E., Konanui, J., Turner, H. (2015). Contemporary Pacific and Western perspectives on kawa (*Piper methysticum*) toxicology. *Fitoterapia, 100*, 56–67.

SPC (2001). Pacific kava: a producer's guide. Secretariat of the Pacific Community. Suva, Fiji Islands.

ACCEPTED MANUSCRIPT

Teschke, R., Sarris, J., Lebot, V. (2011). Kava hepatotoxicity solution: A six-point plan for new kava standardization. *Phytomedicine, 18*, 96-103.

VandenBroucke, H., Mournet, P., Malapa, R., Glaszmann, J.C., Chair, H., Lebot, V. (2015). Comparative analysis of genetic variation in kava (*Piper methysticum*) assessed by SSR and DaRT reveals zygotic foundation and clonal diversification. *Genome, 58*, 1-11.

Van den Dool, H., & Kratz, P.D. (1963). A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography. *Journal of Chromatography A, 11*, 463–471.

Vanuatu Legislation (2002). *Kava Act*. http://www.paclii.org/vu/legis/num_act/toc-K.html (accessed 08.29.10).

Wang, J., Qu, W., Jun, S., Bittenbender, H.C., Li, Q.X. (2010). Rapid determination of six kavalactones in kava root and rhizome samples using Fourier transform infrared spectroscopy and multivariate analysis in comparison with gas chromatography. *Analytical Methods, 2*, 492–498.

Wang, J., Qu, W., Jun, S., Li, Q.X., Bittenbender, H.C. (2013). Kavalactone content and chemotype of kava beverages prepared from roots and rhizomes of Isa and Mahakea varieties and extraction efficiency of kavalactones using different solvents. *Journal of Food Science and Technology, 52*, 1164-1169.

WHO (World Health Organization) (2007). Assessments of the risk of hepatotoxicity with kava products. WHO Document Production Services: Geneva.

Wu, D., Yu, L., Nair, M.G., DeWitt, D.L., Ramsewak, R.S. (2002). Cyclooxygenase enzyme inhibitory compounds with antioxidant activities from *Piper methysticum* (kava kava) roots. *Phytomedicine, 9*, 41-47.

Xuan, T.D., Yuichi, O., Junko, C., Eiji, T., Hiroyuki, T., Mitsuhiro, M., Khan, T.D., Hong, N.H.H. (2003). Kava root (*Piper methysticum* L.) as a potential natural herbicide and fungicide. *Crop Protection, 22*, 873–881.

ACCEPTED MANUSCRIPT

Xuan, T.D., Fukuta, A.A., Wie, A.C., Elzaawely, A.A., Khanh, T.D., Tawata, S. (2008). Efficacy of extracting solvents to chemical compounds of kava (*Piper methysticum*) root. *Journal of Natural Medicine, 62*, 188-194.

Zhou, P., Gross, S., Liu, J.H., Yu, B.Y., Feng, L.L., Nolta, J., Sharma, V., Piwnica-Worms, D., Qiu, S. (2010). Flavokawain B, the hepatotoxic constituent from kava root, induces GSH-sensitive oxidative stress through modulation of IKK/NF-B and MAPK signaling pathways. *FASEB Journal, 24*, 4722-4732.

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Figure 1. Major constituents of kava. The structures of the 6 major kavalactones (A) and flavokavains (B) of kava roots are shown. Abbreviations used in the text are placed in parenthesis after each name.

Figure 2. Colors of noble kava 'Borogu' (A) and wichmannii kava 'Wichmannii' (B) root powder extracts. Dry root powder was extracted with water (1), acetone (2), chloroform (3), methanol (4), ethanol (5), hexane (6) or diethyl ether (7).

Figure 3. TLC analysis of root powder extracts. Dry root powder of wichmannii kava 'Wichmannii' (lanes 1–7') or noble kava 'Borogu' (lanes 8–14) was extracted with water (1,8), acetone (2,9), chloroform (3,10), methanol (4,11), ethanol (5,12), hexane (6,13), diethyl ether (7,14) or diethyl ether followed by acetone (7'). Seventy µL of each extract were loaded except for water extracts where only 30 µL were spotted. Plates were visualized with white light (A) and under illumination at 365 nm (B).

Figure 4. Analyses of diethyl ether extracts by GC/MS (A) and LC/MS (B, C). PCA plots obtained for the peaks areas of all annotated substances by GC/MS (A) and LS/MS (B). Histograms of means +/- SD of peak areas of all six major KLs and two major FKs (C). Peak areas were estimated on extracted ion chromatograms of these substances after LC/MS analysis of crude extracts. For each of these substances, pairwise Wilcoxon tests were conducted to group kava classes into statistical clusters (letters above bars) at $p < 0.05$. Seven noble, three two-day and five wichmannii cultivars were analyzed.

Figure 5. Typical absorption spectra of a diethyl ether extract of the roots of noble, two-day and wichmannii cultivars. Absorbance spectra of a noble ('Ni Kawa Pia' – circles), a two-day ('Twoday' – squares) and a wichmannii ('Sini Bo' – triangles) were recorded from 210 nm to 430 nm. Absorbances were zeroed against diethyl ether at each wavelength.

Figure 6. Multivariate analysis of UV/VIS absorbance spectra of kava cultivars. PCA plot of absorbance values obtained every 10 nm from 210 nm to 420 nm on kava cultivar root diethyl ether extracts zeroed against diethyl ether and normalized against the extract absorbance at 350 nm. Seven noble, three two-day and five wichmannii cultivars were analyzed.

Figure 7. Ratio of the absorbance at 250 nm over the one at 290 nm of kava cultivar diethyl ether extracts. Kava cultivars were grouped according to their genetic group and bars represent means +/- SD of ratio values for each class.

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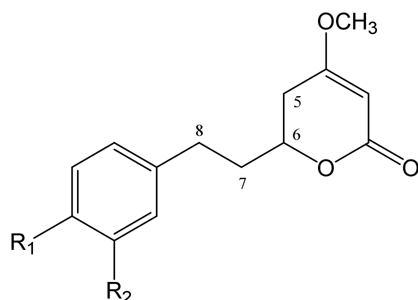
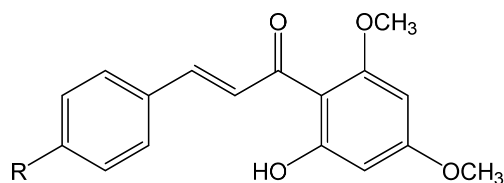
Figure 1

A



Kavalactone (KL)	R1	R2	C5-C6	C7-C8
Methysticin (M)	OCH_2O			=
Dihydromethysticin (DHM)	OCH_2O			
Kavain (K)				=
7,8-Dihydrokavain (DHK)				
Desmethoxyyangonin (DMY)			=	=
Yangonin (Y)	OCH_3		=	=

B



Flavokavain (FK)	R
Flavokavin A (FKA)	OCH_3
Flavokavin B (FKB)	

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Figure 2

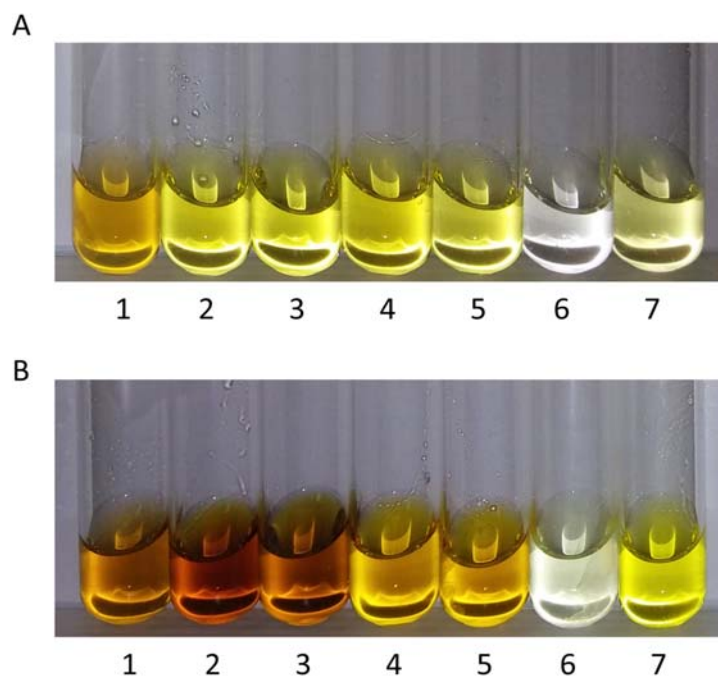

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Figure 3

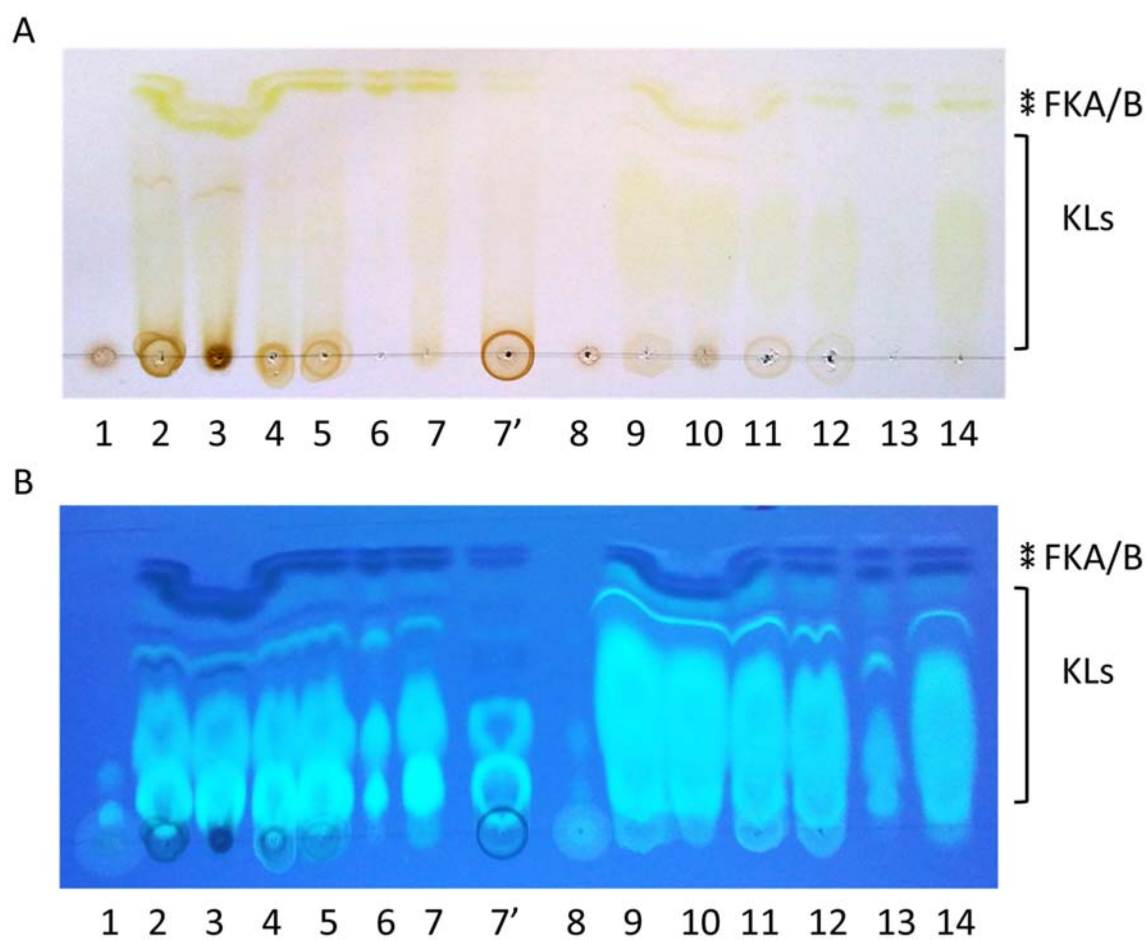

Figure 4

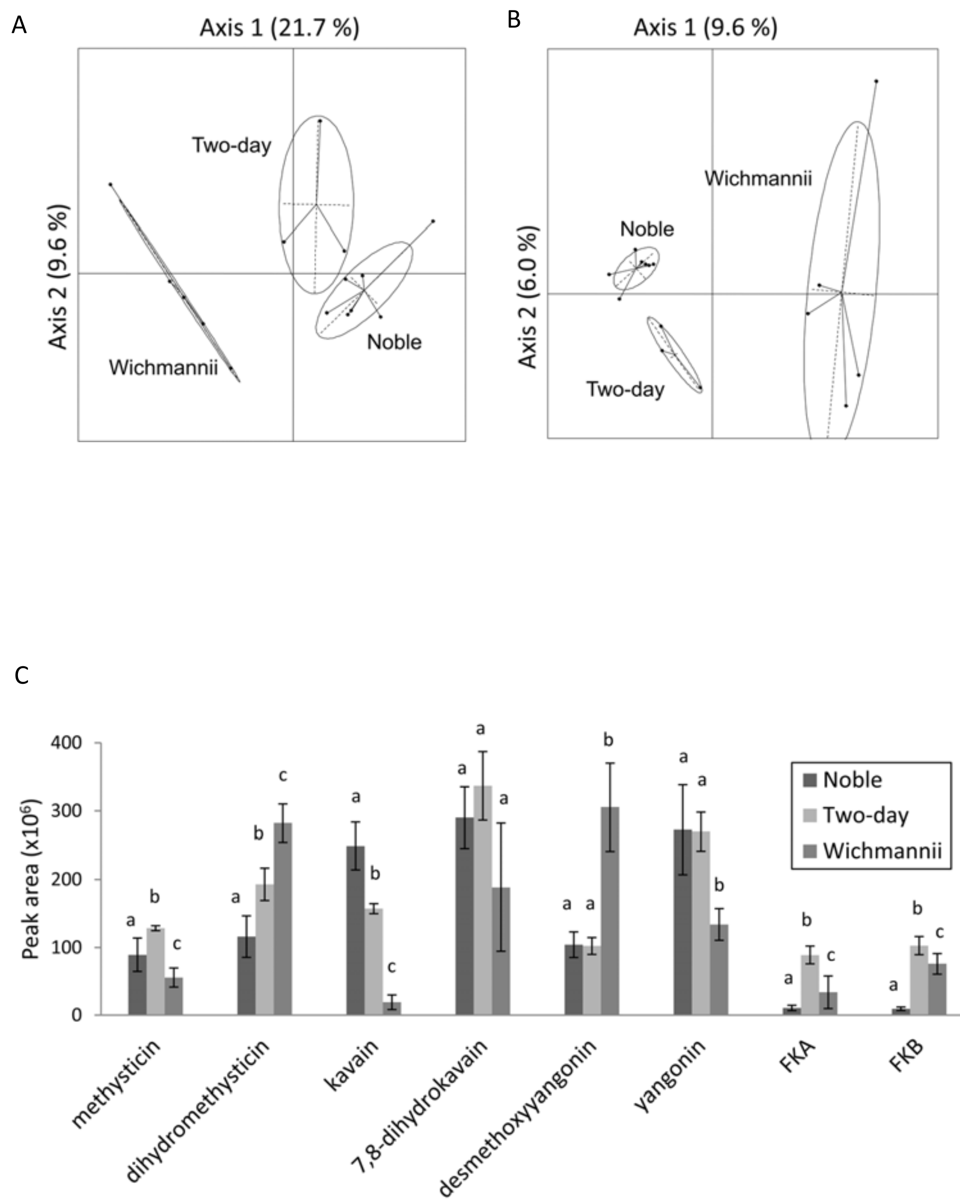

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Figure 5

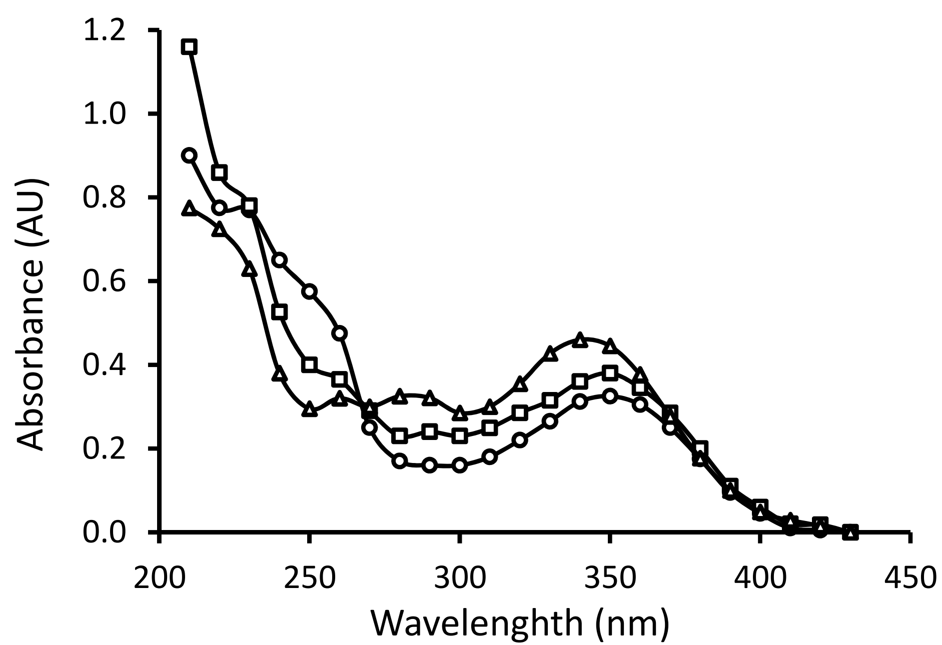

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Figure 6

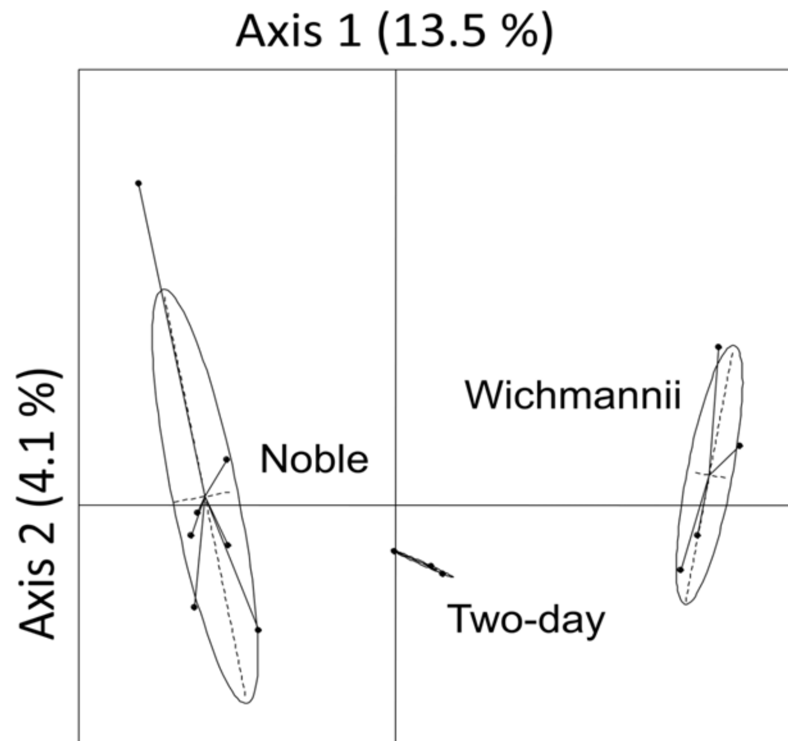

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Figure 7

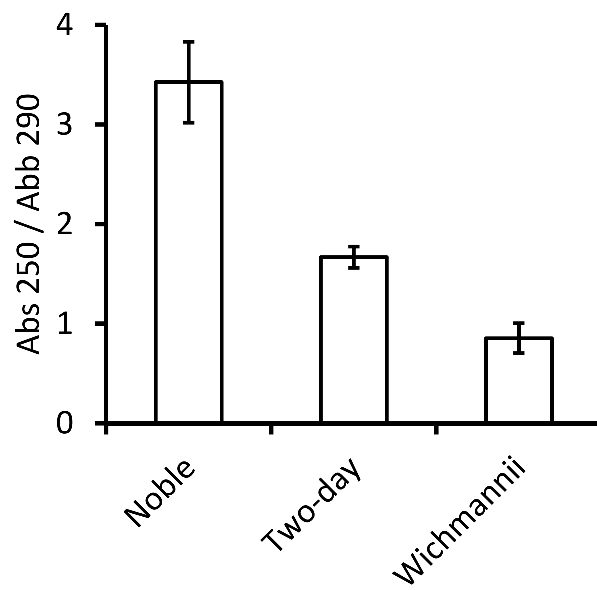

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Table 1

Major KLs and FKs detected during GC/MS analysis.

Nb[a]	RT[b] (min)	RI[c]	mass (*m/z*)	name (abbreviation)
40	45.03	2163	232	7,8-dihydrokavain (DHK)
43	47.42	2272	230	kavain (K)
48	49.14	2355	228	desmethoxyyangonin (DMY)
52	53.37	2569	276	dihydromethysticin (DHM)
53	54.59	2632	284	flavokavain B (FKB)
55	55.36	2675	258	yangonin (Y)
58	60.29	2959	314	flavokavain A (FKA)

[a] Order or appearance on chromatogram among annotated substances; [b] Retention times (RT) as measured during GC-MS analysis; [c] Kovats' retention indices (RI) calculated by comparison to n-alkanes according to van den Dool and Kratz, 1963

Table 2

Substances present during LC/MS analysis.

Nb[a]	RT[b] (min)	λmax (nm)	$[M+H]^+$ (*m/z*)	name (abbreviation)[b]
1	1.756	220, 266, 306	275.0911	methysticin (M)
2	1.830	235, 286	277.1066	dihydromethysticin (DHM)
3	2.088	225	263.1276	
4	2.105	243	231.1013	kavain (K)
5	2.263	236	233.1168	7,8-dihydrokavain (DHK)
6	2.454	288, 334	259.0962	
7	2.837	230, 256, 342	229.0855	desmethoxyyangonin (DMY)
8	2.870	223, 260, 354	259.0963	yangonin (Y)
9	3.086	282	315.1226	
10	3.227	284	285.1117	
11	3.510	-	387.1793	
12	5.09	284	487.1749	
13	5.132	286	457.1642	
14	5.489	346	271.0958	
15	5.531	290, 345	301.1065	
16	8.167	-	457.1636	
17	8.391	288, 332sh	271.0962	
18	8.399	288, 332sh	259.0961	
19	8.566	252	229.0856	
20	8.582	255	259.0967	
21	10.362	364	315.1227	flavokavain A (FKA)
22	10.545	342	285.1117	flavokavain B (FKB)

[a] Order of elution; [b] Identified by comparison with standards. Elution order and absorption maxima similar to previous reports (*Lebot et al.,* 2016; Meissner and Häberlein, 2005).