Journal of Applied Research on Medicinal and Aromatic Plants 16 (2020) 100235

Contents lists available at ScienceDirect

Journal of Applied Research on Medicinal and Aromatic Plants

journal homepage: www.elsevier.com/locate/jarmap

Rapid differentiation of *Piper methysticum* (kava) plant parts using single point and imaging vibrational spectroscopy

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ARTICLE INFO

Keywords:
Piper methysticum
Kava
Spectroscopy
Hyperspectral imaging
Kavalactones
Flavokawains

ABSTRACT

Piper methysticum Forst., commonly referred to as kava, has been used medicinally and recreationally by inhabitants of the South Pacific Islands for centuries. Kavalactones present in roots and aerial parts are regarded as the bioactive compounds responsible for the relaxant effects, and for mitigating anxiety and stress-related conditions. The development of pharmaceutical products containing root extracts led to a boom in kava sales in Europe in 1998. However, reported cases of toxicity led to the subsequent banning of kava products in several countries. This study was initiated to develop rapid, robust and alternative spectroscopic methods for quality control that can be implemented at the point of export, to authenticate the use of kava roots as legislated by the Vanuatu Amended Kava Act no. 6 (2015). Roots, peeled stems, and stump peelings samples (n = 47) were sourced from Fiji, Hawaii, Samoa, the Solomon Islands and Tonga. The sample extracts were analysed using ultra performance liquid chromatography coupled to a photodiode array detector and mass spectrometer (UHPLC-PDA/MS), while powdered material was analysed using spectroscopic techniques. These included single-point (near-infrared (NIR) and mid-infrared (MIR) spectroscopy), as well as an imaging (hyperspectral imaging). Principal component analysis of both the raw UPLC-MS and the quantitative UPLC-PDA data revealed chemical differences between the root and non-root samples. Kavain, methysticin and yangonin were identified as the compounds largely responsible for the chemical differences between the plant parts. Discriminant analysis models (OPLS-DA and PLS-DA) were developed for all the techniques, to reliably discriminate kava roots from non-roots. All the discriminant models indicated a good prediction ability ($Q^2X_{Cum} \geq 60$ %) and were successfully used to accurately identify external roots and non-root samples. However, hyperspectral imaging yielded superior results, with a prediction ability above 90 %. This technique can be automated and is capable of continuously scanning multiple samples, making it ideal for quality control.

1. Introduction

Piper methysticum Forst. (family Piperaceae), known as '*kava*', '*yangona*' or '*awa*', is native to the Polynesian (Hawaii, Samoa and Tonga) and Melanesian (Fiji, the Solomon Islands and Vanuatu) islands (Turner, 1986; Gautz et al., 2006). The shrub plays an important cultural role in the lives of the South Pacific islanders. A relaxing and tranquilizing drink (also referred to as kava) is prepared by maceration of the roots or peeled rhizome with water or coconut water (Whittaker et al., 2008). The drink forms part of traditional ceremonies, is sold recreationally in bars, and is often consumed before meals in homes (Davis and Brown, 1999; Rychetnik and Madronio, 2011). Traditional kava was used for hundreds of years without any recorded adverse effects (Whitton et al., 2003). It was used medicinally in Europe since the late 18th Century, but became popular in the 1990s, when pharmaceutical products designed to treat anxiety were developed from ethanolic and acetonic extracts of the root (Baker, 2011).

Several kavalactones contribute to the relaxing and sedating properties of the shrub (Schmidt and Molnar, 2002; Xuan et al., 2006). To date, 18 kavalactones have been isolated from kava and identified. However, six of these make up more than 95 % of the kavalactone content of root and rhizome extracts, and are therefore regarded as biomarkers for the species (Lebot and Levesque, 1996). A coding system that involves the assignment of specific numbers to each of the

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https://doi.org/10.1016/j.jarmap.2019.100235
Received 31 July 2019; Received in revised form 26 November 2019; Accepted 26 November 2019
Available online 28 November 2019

biomarkers (desmethoxyyangonin [1], dihydrokavain [2], yangonin [3], kavain [4], dihydromethysticin [5] and methysticin [6]) was developed by Lebot and Levesque (1996), to assign characteristic kava cultivar signatures (chemotypes). Each chemotype is characterised using the concentrations of the kavalactones determined in the extract, by listing the numbers in decreasing order of abundance. For example, the code 426315 indicates that kavain is the most abundant, followed by dihydrokavain and methysticin, while dihydromethysticin is the least abundant in the extract. However, the locals use classification systems, which vary from country to country, based on the morphology of the plant and the physiological effects of the root extracts (Davis and Brown, 1999). Legislation (Amended Kava Act No. 6 of 2015) was passed in Vanuatu that implements an alternative classification system for different varieties as follows: *Noble* kava (cultivars that have been safely used socially for millennia) *versus* "narafala kava" (any variety other than *Noble* kava). Narafala kava includes the former definitions as "Medicinal Kava" (cultivars that are used medicinally), *Two-day/Tudei* kava (cultivars that exert a strong effect on the consumer and causes hangovers lasting for more than 24 h), and *Wichmannii* kava (*Piper wichmannii* C. DC.). *Noble* cultivars are characterised by high concentrations of kavain, dihydrokavain and methysticin, while non-noble kava typically have a low kavain and a high dihydromethysticin content (Lebot and Levesque, 1996).

Kava was implicated in cases of liver toxicity that occurred between 1999 and 2001, resulting in kava being prohibited in some European countries (Schmidt et al., 2002). A ban was instated in Germany in June 2002, where after several countries (England, Australia, and Canada) followed suit, despite no cases of toxicity having been reported in those regions (Blumenthal, 2002; Clouatre, 2004; Teschke et al., 2011a). At the time, the United States Food and Drug Administration (USFDA) issued a warning to physicians and patients regarding the risk of using kava products (Blumenthal, 2002; Clouatre, 2004). However, scientists argued that the ban had been instituted with undue haste and without proper scrutiny of the evidence (Blumenthal, 2002). Indeed, after a thorough evaluation of the reported cases, it was concluded that in only case, toxicity had ensued after intake of the correct dosage of a kava product (Blumenthal, 2002; Gruenwald et al., 2003; Clouatre, 2004). In 2014 and 2015, German courts ruled that kava could be allowed back into the German market, however, only as a prescribed medication (Kuchta et al., 2015).

Since 2002, many steps have been taken to improve and standardise the quality control of kava, including the promulgation of the Vanuatu Kava Act, quality regulations in Vanuatu (Vanuatu National Standard for Kava Export), Fiji (Fiji Kava quality manual and Fiji Kava *Piper methysticum* Forst f. Standard 2017) and Samoa, (Food 'Awa Regulations 2018 and Samoa 'Ava Standard 2028). Moreover, there was an acceptance of a regional Pacific Codex Alimentarius standard for kava (Teschke and Lebot, 2011). Several researchers proposed standardisation codes, and a six-point standardisation plan to improve the quality and safety of kava was proposed (Teschke and Lebot, 2011; Teschke et al., 2011b). A quick test based on the colour level of an acetone extract was suggested (Siméoni and Lebot, 2014) and is currently applied in the testing of export material of kava in Vanuatu. Furthermore, an high performance thin layer chromatography (HPTLC) method was described for distinguishing between *Noble* and non-noble kava varieties, based on the distinctly higher flavokawain B contents in non-noble varieties (Lebot et al., 2014, 2019).

Developed countries, including Germany, enforce quality control standards and regulations on kava imports, which pertained to dosage, duration of use, indication, contra-indications, and plant part to use (Teschke et al., 2011b). High performance liquid chromatography (HPLC) is accepted as the standard technique for analysis. However, in many cases, comprehensive quality standards are not maintained by the exporting country. Sophisticated analytical instrumentation, such as high- or ultra performance liquid chromatography (HPLC or UPLC), and skilled personnel are not readily available. Quality control is therefore reliant on visual inspection.

Kava is mainly exported in the form of dry roots, peeled rhizomes or in powdered form (Davis and Brown, 1999). The use of aerial parts and specifically unpeeled stem material or peelings in kava preparation poses a risk, since they may contain pipermethystine, a potentially toxic compound that may have been involved in the reported kava toxicity discussed (Teschke et al., 2011a). It has been speculated that during the kava boom (1998–2000), exporters may have compensated for the shortage of preferred parts for export (dry roots/peeled rhizome) by using aerial parts of the shrub, including the leaves, to cope with demand (Lim et al., 2007). This hypothesis was refuted in the case of kava imports to Germany (Kuchta et al., 2015; Lechtenberg et al., 2018), however, it may still apply to some kava exports from Vanuatu. The development of reliable, rapid and cost-effective techniques that can be applied to verify the plant part of powdered raw material during quality control, prior to export, would make a valuable contribution to ensuring the safety of kava products.

Although HPLC is the technique of choice for the determination of kavalactones and Flavokavains (chalcones present in low concentrations in the kava plant) (Lebot, 2006), it is not ideal for rapid throughput, since the sample preparation is time-consuming (Wang et al., 2013), and the climatic conditions in the South Pacific call for a more robust and easy to use methodology. Infrared spectroscopy and hyperspectral imaging (HSI) are emerging as suitable alternatives for quality control in the herbal and food industries (Gowen et al., 2007; Lasme et al., 2008). Spectroscopic techniques are faster and allow the analysis of solid matrices, without sample pre-treatment other than grinding and sieving. In addition, HSI contributes visual (spatial) data for easy interpretation (Lasme et al., 2008). The aim of this study was to develop robust vibrational spectroscopy techniques that can be used to distinguish kava roots from other plant parts, once they are in the powdered form.

2. Materials and methods

2.1. Reference compounds and reagents

Kava biomarkers methysticin, dihydromethysticin, yangonin, desmethoxyyangonin and dihydrokavain (≥95 % purity) were purchased from Phytolab (Vestenbergsgreuth, Germany), and kavain was obtained from Sigma-Aldrich (St. Louis, Missouri, USA). Flavokawains (A and B) (≥95 % purity) were purchased from Chromadex (California, USA). Acetone, 1.4-dioxane, dichloromethane, ethyl acetate, ethanol, *n*-hexane and methanol (AR grade) were supplied by Merck (Darmstadt, Germany). Ultra gradient solvents (acetonitrile, formic acid, methanol) were purchased from Romil (Cambridge, England).

2.2. Samples and sample preparation

Kava roots (R), peeled stems (ST), and stump peelings (SP) were obtained in collaboration with HerbResearch Germany. The study included a total of 47 samples from five countries: Fiji = 20 (R = 16 and SP = 4), Hawaii = 12 (R = 8 and SP = 4), Samoa = 3 (R = 2 and SP = 1), Solomon Islands = 1 (R = 1), and Tonga = 11 (R = 5, ST = 6). The samples were ground to a fine powder.

For UPLC, 2.0 g of each sample was extracted by sonication with 40 ml acetone for 30 min, while the temperature was maintained below 40 °C. The mixture was left to settle, and the supernatant was decanted. The solid was re-extracted twice as described, where after the combined extracts were filtered and concentrated to a volume of less than 10 mL, using a Büchi rotary evaporator (Model RT15), at 40 °C and 70 rpm. The concentrate was subsequently transferred to a pre-weighed glass tube and dried using a Genevac EZ-2 Plus centrifugal evaporator. After drying, the extract yields were determined by weighing. The UPLC sample preparation involved dissolving the crude extracts in ultra gradient methanol, to obtain a final concentration of 1 mg/mL. Finally,

the solutions were filtered through Clarinet syringe filters (0.22 µm) into certified vials with pre-slit PTFE/silicone caps.

Sample preparation for vibrational spectroscopy only involved sieving of the powdered samples through a 250 µm mesh (A.S.T.M.E 11, Pascal Engineering Co, Ltd, Sussex, England).

2.3. Sample analysis using ultra performance liquid chromatography-photodiode array detection/mass spectrometry (UPLC-PDA/MS)

2.3.1. Method development

An analytical method for the simultaneous determination of the six kavalactone biomarkers and two flavokawains was developed and validated using a Waters Acquity UPLC system. The system was coupled to a Waters photodiode array (PDA) detector (Milford, Massachusetts, USA). A 1 µL (full loop) volume of each sample was analysed. Separation of the sample components was accomplished on a Waters Acquity UPLC BEH C18 column (150 mm × 2.1 mm i.d., 1.7 µm particle size), maintained at 30 °C. The mobile phase comprised 0.1 % formic acid in water (Solvent A) and 90 % acetonitrile (Solvent B), at a flow rate of 0.3 mL/min. Various gradients were tested to achieve the best resolution of the eight standards. Gradient elution was applied as follow: 55 % A:45 % B, held for 2 min, to 42 % A:58 % B within 8 min, changed to 10 % A:90 % B over 1 min, held for 2 min, and back to the initial ratio in 0.5 min. The total running time was 13.5 min.

The UPLC system was simultaneously coupled to a Xevo G2 quadrupole Time-of-Flight (qToF) mass spectrometer (MS) (Waters, USA). Both positive and negative electrospray ionisation modes were evaluated. The positive mode was selected for sample analysis since it yielded the highest detector responses for the analytes. Nitrogen at a flow rate of 550 l/h was used as the desolvation gas, and the desolvation temperature was set to 450 °C. A source temperature of 100 °C was maintained. The capillary and cone voltages were set to 3500 and 45 V, respectively. Data were collected between *m/z* 100 and 1500 and processed by Masslynx 4.1 chromatographic software.

2.3.2. Method validation

Method validation was carried out according to the guidelines of the International Conference for Harmonisation (ICH, 2006). Calibration curves were developed from the UPLC-PDA results generated from individual calibration standards, prepared as eight concentrations of each of the eight reference compounds over the range 0.500–100 µg/mL. The limits of detection (LOD) and quantification (LOQ) were established using the average standard deviation of the response and the slope, following regression analysis using Microsoft Excel 2013 (Miller and Miller, 2010). Intra- and inter-day precision were determined by analysing one concentration level (10 µg/mL) of all of the analytes in triplicate. The analyses were carried out twice on the first day, and once on each of the second and third days. The percentage relative standard deviation (%RSD) was used as a measure of precision. Method accuracy was determined by calculating the recoveries of the eight compounds, after spiking portions of a sample containing known concentrations of the analytes. Standard addition was done in triplicate at three concentration levels to yield final spiked concentrations of 1.25, 12.5 and 50.0 µg/ml of each analyte in the samples. The percentage recoveries were calculated as the concentration recovered after spiking divided by the sum of the extract and spike concentrations.

2.3.3. Chemometric analysis of the data

A principal component analysis (PCA) model was created after uploading the raw UPLC-MS data from Excel into SIMCA 14 (Umetrics AB, Sweden) software. Models were constructed using data from 43 samples, representing 30 root, 5 peeled stems and 8 stump peelings samples. The effect on the resulting model of applying different scaling methods *i.e.* Center (Ctr), Pareto (Par) and Unit Variance (UV) to the dataset, was investigated by comparing the PCA model statistics obtained. The dataset yielding the best statistics was retained to further develop an orthogonal projection to latent structures-discriminant analysis (OPLS-DA) model. Four randomly selected samples, consisting of two roots, one peeled stem, and one stump peeling sample, were not included in the models, to be used later for external validation purposes.

Table 1
Results obtained for the UPLC-PDA method validation including linearity, limits of detection (LOD) and quantification (LOQ), and percentage recoveries for each of the six kavalactone biomarkers and flavokawains A and B.

Compounds	Coefficient of determination (R^2)	LOD µg/mL	LOQ µg/mL	Recovery Level	_%
Methysticin				1.25	103
	0.9994	0.268	0.964	12.5	104
				50.0	101
Dihydromethysticin				1.25	103
	0.9992	0.289	0.964	12.5	104
				50.0	98.7
Kavain				1.25	103
	0.9996	0.424	1.41	12.5	104
				50.0	102
Dihydrokavain				1.25	103
	0.9998	0.437	1.46	12.5	104
				50.0	101
Yangonin				1.25	103
	0.9996	0.247	0.823	12.5	104
				50.0	99.1
Desmethoxyyangonin				1.25	101
	0.9983	0.286	0.953	12.5	104
				50.0	98.4
Flavokawain A				1.25	104
	0.9991	0.218	0.726	12.5	96.0
				50.0	99.0
Flavokawain B				1.25	107
	0.9991	0.218	0.726	12.5	101
				50.0	94.8

2.4. Near- and mid-infrared spectroscopy

2.4.1. Acquisition of spectra

Powdered samples (roots, stump peelings and peeled stems) were placed in vials and analysed using an NIR Flex N500 solid cell spectrometer (Büchi Labortechnik AG, Flawil, Switzerland). Spectra were collected over the range $10\,000 - 4000\ cm^{-1}$ in reflectance mode, using the NIR WARE 1.2 software. For each sample, spectra were recorded three times, and exported to Microsoft Excel 2013. The software was used to calculate the averages of the replicates for the 47 samples in preparation for chemometric analysis using SIMCA 14.

An Alpha-P Bruker spectrometer (Bruker OPTIK GmbH, Ettlingen, Germany) with an attenuated total reflectance (ATR) diamond crystal was used to record the mid-infrared (MIR) spectra of the powdered samples. The spectra were captured with OPUS 6.5 software. Thirty-two scans were recorded in triplicate at a spectral resolution of $500 - 4000\ cm^{-1}$. After exporting the data to Microsoft Excel 2013, the averages of the replicates for the 47 samples were calculated in preparation for chemometric analysis in SIMCA 14.

2.4.2. Chemometric analysis of the data

Various scaling methods and spectral pre-processing methods (Multiple Scatter Correction (MSC), Standard Normal Variate (SNV), and Spectral Derivatives (1^{st} and 2^{nd}) were applied to the spectroscopic data, during the construction of individual PCA models. Spectral pre-processing algorithms are used to remove baseline shifts that may occur due to variations in temperature, humidity, particle size of the powders, and the instability of the instrument (Blanco & Villarroya, 2002; Reich, 2005; Rinnan et al., 2009; Barbin et al., 2012). The PCA model yielding the best model statistics was used to develop an OPLS-DA model. Finally the OPLS-DA model was validated by predicting the plant parts

Table 2
Intra- and intraday precision of the UPLC-PDA method developed for the determination of the six kavalactone biomarkers and flavokawains A and B.

Compounds	M	DHM	K	DHK	Y	DMY	FKA	FKB
Day 1 Intra-day								
Interval 1 (μg/mL)	10.3	10.5	10.1	10.3	10.4	9.27	11.1	10.6
%RSD	± 0.00	± 0.00	± 0.01	± 0.03	± 0.00	± 0.01	± 0.00	± 0.00
Interval 2 (μg/mL)	10.3	10.4	10.1	10.2	10.4	9.30	11.1	10.6
%RSD	± 0.01	± 0.01	± 0.01	± 0.01	± 0.00	± 0.00	± 0.01	± 0.01
Inter-day								
Day 2 (μg/mL)	10.3	10.5	10.1	10.3	10.4	9.28	11.1	10.6
%RSD	± 0.39	± 0.80	± 0.63	± 1.83	± 0.00	± 0.44	± 0.37	± 0.38
Day 3 (μg/mL)	10.3	10.5	10.0	10.2	10.5	9.37	11.1	10.5
%RSD	± 0.76	± 1.26	± 1.23	± 1.90	± 1.39	± 1.41	± 0.60	± 1.46

M = methysticin, DHM = dihydromethysticin, K = kavain, DHK = dihydrokavain, Y = yangonin, DMY = desmethoxyyangonin. KVLs = kavalactones, FKA = flavokawain A and FKB = flavokawain B, ND = not detected.

Table 3
Average concentrations of kavalactones and flavokawains A and B in various kava plant parts of different origins.

	Compounds	M	DHM	K	DHK	Y	DMY	Total KVLs	FKA	FKB
	Fiji									
Roots	Mean	9.44	3.31	16.1	9.33	3.71	5.08	46.9	0.242	0.264
	Max	16.8	4.84	23.0	13.9	6.07	11.0	64.5	0.377	0.504
	Min	6.10	2.15	9.41	5.70	1.85	2.76	29.2	0.120	0.130
	Std	± 2.88	± 0.825	± 3.83	± 2.05	± 1.31	± 2.05	± 10.9	± 0.073	± 0.107
Stump peelings	Mean	11.7	8.97	16.2	20.2	3.84	4.07	65.0	1.09	0.880
	Max	15.8	17.3	25.8	42.2	8.04	5.94	110	2.75	2.21
	Min	8.56	5.01	12.7	11.5	1.89	1.21	46.2	0.383	0.403
	Std	± 3.35	± 5.66	± 6.42	± 14.7	± 2.83	± 2.02	± 30.4	± 1.12	± 0.890
	Hawaii									
Roots	Mean	13.0	4.82	21.6	12.3	6.53	7.61	65.9	0.460	0.284
	Max	16.4	6.65	29.9	19.4	12.2	10.2	83.6	0.992	0.665
	Min	9.81	3.04	12.9	7.14	2.72	5.07	46.7	0.245	0.138
	Std	± 2.00	± 1.39	± 5.07	± 4.22	± 2.81	± 1.91	± 13.7	± 0.239	± 0.171
Stump peelings	Mean	13.5	12.1	16.9	27.4	5.54	10.1	85.6	1.62	1.18
	Max	16.3	15.1	22.4	34.8	6.29	11.1	104	1.93	1.47
	Min	11.1	8.90	6.88	19.9	4.63	7.79	68.6	1.23	0.856
	Std	± 2.34	± 2.63	± 7.27	± 6.07	± 0.870	± 1.52	± 17.6	± 0.304	± 0.255
	Tonga									
Roots	Mean	8.50	4.00	13.4	9.97	3.42	5.91	45.2	0.413	0.356
	Max	12.1	7.06	20.1	16.1	4.48	7.93	67.8	1.26	1.05
	Min	5.85	2.64	10.5	6.59	2.30	4.10	35.1	0.129	0.102
	Std	± 2.16	± 1.65	± 3.44	± 3.43	± 1.09	± 1.46	± 8.20	± 0.429	± 0.362
Peeled stems	Mean	1.31	1.14	2.10	3.20	0.519	0.946	9.22	0.191	0.149
	Max	2.05	1.46	3.76	6.01	0.958	1.72	14.8	0.345	0.256
	Min	0.498	0.276	0.698	1.57	0.216	0.407	4.45	0.108	0.068
	Std	± 0.543	± 0.454	± 1.00	± 1.50	± 0.245	± 0.458	± 3.38	± 0.095	± 0.091
	Samoa									
Roots	Mean	7.94	2.30	6.39	7.64	6.04	3.81	34.1	0.174	0.190
	Max	9.37	2.79	10.5	9.98	8.22	4.88	45.8	0.245	0.231
	Min	6.51	1.81	2.23	5.31	3.87	2.75	22.5	0.103	0.148
	Std	± 2.02	± 0.692	± 5.88	± 3.30	± 3.07	± 1.51	± 16.5	± 0.101	± 0.058
Stump peelings	True values (one sample)	4.24	2.97	9.59	8.99	1.72	2.83	30.3	0.473	0.348
	The Solomon Islands									
Roots	True values (one sample)	4.90	2.83	27.60	20.20	5.92	5.90	67.3	0.353	0.272

M = methysticin, DHM = dihydromethysticin, K = kavain, DHK = dihydrokavain, Y = yangonin, DMY = desmethoxyyangonin, KVLs = kavalactones, FKA = flavokawain A and FKB = flavokawain B, Max = highest, Min = lowest and Std = standard deviation.

represented by the selected external samples.

2.5. Hyperspectral imaging

2.5.1. Spectral acquisition

A SisuCHEMA shortwave infrared (SWIR) hyperspectral imaging camera (Specim, Spectral Imaging Ltd., Oulu, Finland) with a spectral range of 920 − 2514 nm, controlled by Chemadaq (Version 3.62.183.19) software, was used to capture hyperspectral images with a high magnification lens (field of view: 50 mm; spatial resolution: 0.3 μm). The system consisted of an imaging spectrograph coupled to a two-dimensional array mercury–cadmium–telluride (HgCdTe) detector. White and black references were captured prior to image acquisition, to minimise variations in sample illumination.

A clean sheet of paper was placed on the mobile plane carriage of the instrument. Powdered samples (roots, peeled stems, and stump peelings) were randomly positioned on the paper, shaped into a circle and scanned. Image acquisition commenced as the samples on the moving carriage entered the field of view. Hyperspectral images (hypercubes) were acquired in diffuse reflectance mode with a pixel size of 256 × 320 and a pixel depth of 14 bits/pixel. Evince® multivariate analysis software version 2.4.0 (UmBio AB, Umea, Sweden) was used to remove the background pixels corresponding to the paper in the image, until only pixels corresponding to the samples remained. Once the

Table 4
UPLC-PDA quantification results comparing kavalactone content (mg/g) between roots and stump peelings of different varieties of kava from various sources.

Plant parts	Variety	M	DHM	K	DHK	Y	DMY	Total KVLs	FKA	FKB
Fiji										
Roots	Vula	6.62	2.95	11.0	7.00	1.85	3.67	33.1	0.204	0.204
Stump peelings		8.56	6.08	12.7	13.5	1.89	5.94	48.7	0.646	0.469
Hawaii										
Roots	Mahakea	9.81	3.43	12.9	7.14	7.26	6.20	46.7	0.256	0.146
Stump peelings		16.3	13.2	22.1	28.5	6.29	10.8	97.3	1.78	1.24
Roots	Moi	13.3	5.86	23.0	14.7	4.76	7.67	69.4	0.462	0.227
Stump peelings		12.1	11.4	6.88	26.6	4.97	10.6	72.4	1.54	1.13
Roots	Nene	16.4	6.45	25.9	16.3	7.57	8.83	81.4	0.385	0.186
Stump peelings		14.4	15.1	22.4	34.8	6.28	11.1	104	1.93	1.47

M = methysticin, DHM = dihydromethysticin, K = kavain, DHK = dihydrokavain, Y = yangonin, DMY = desmethoxyyangonin, KVLs = kavalactones, FKA = flavokawain A and FKB = flavokawain B.

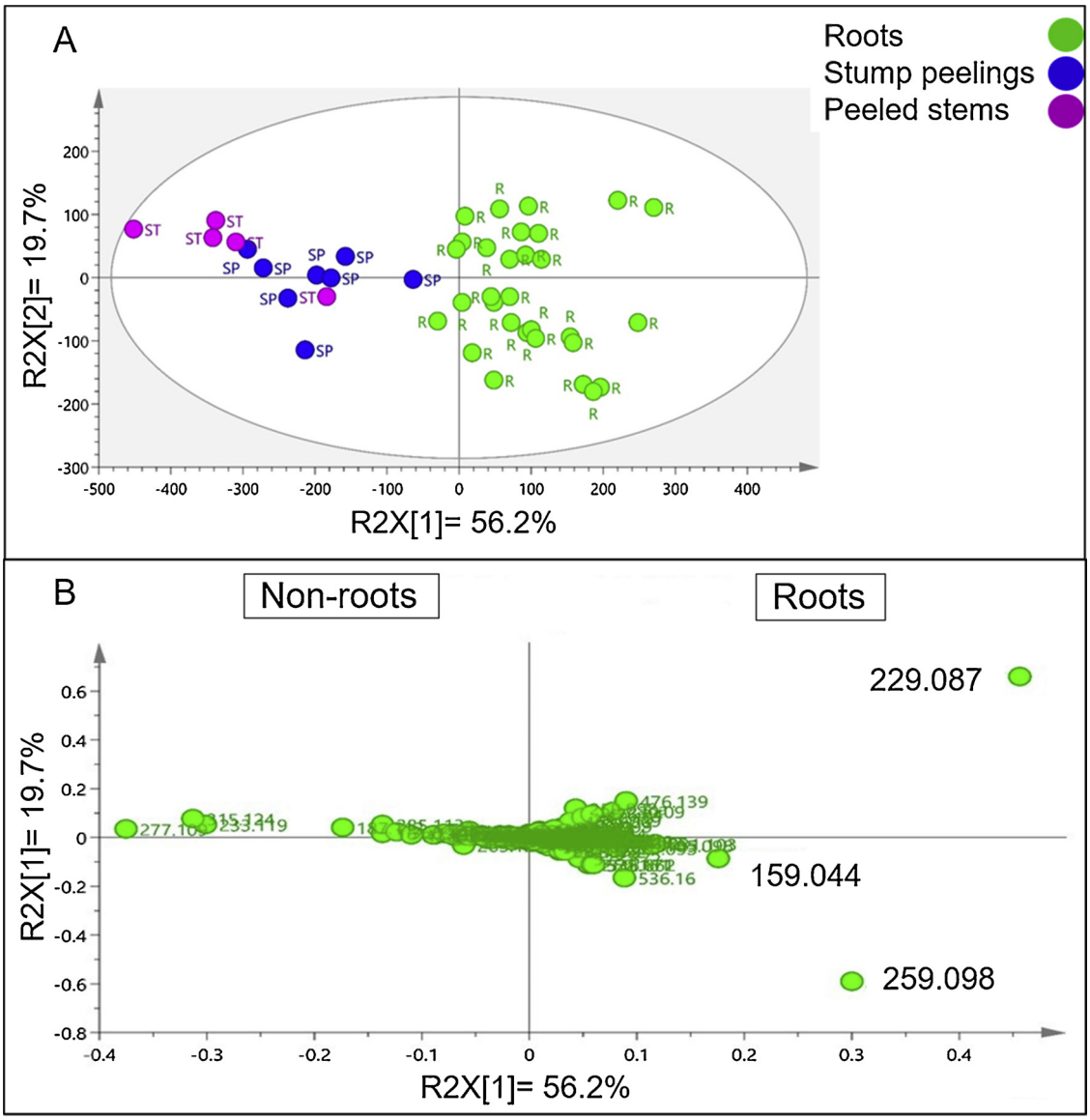

Fig. 1. A) PCA scores plot derived from UPLC-MS data indicating chemical differences between the kava roots, stump peelings and peeled stems B) Corresponding loadings plots indicating mass fragments (at the extreme ends) associated with the differentiation of the plant parts.

undesired pixels had been removed, a PCA model was constructed using the cleaned data. In addition to eliminating the background, the trend tool (multivariate image analysis toolbox (MIA), MATLAB R2018) was applied as it offers a unique option when analysing hypercubes. The 256 wavelengths obtained from HSI usually contains a level of noise that obstructs the chemical variation when using different species or plant parts (Tankeu et al., 2016). The dataset dimension was thereby reduced before constructing the models (PCA and PLS-DA).

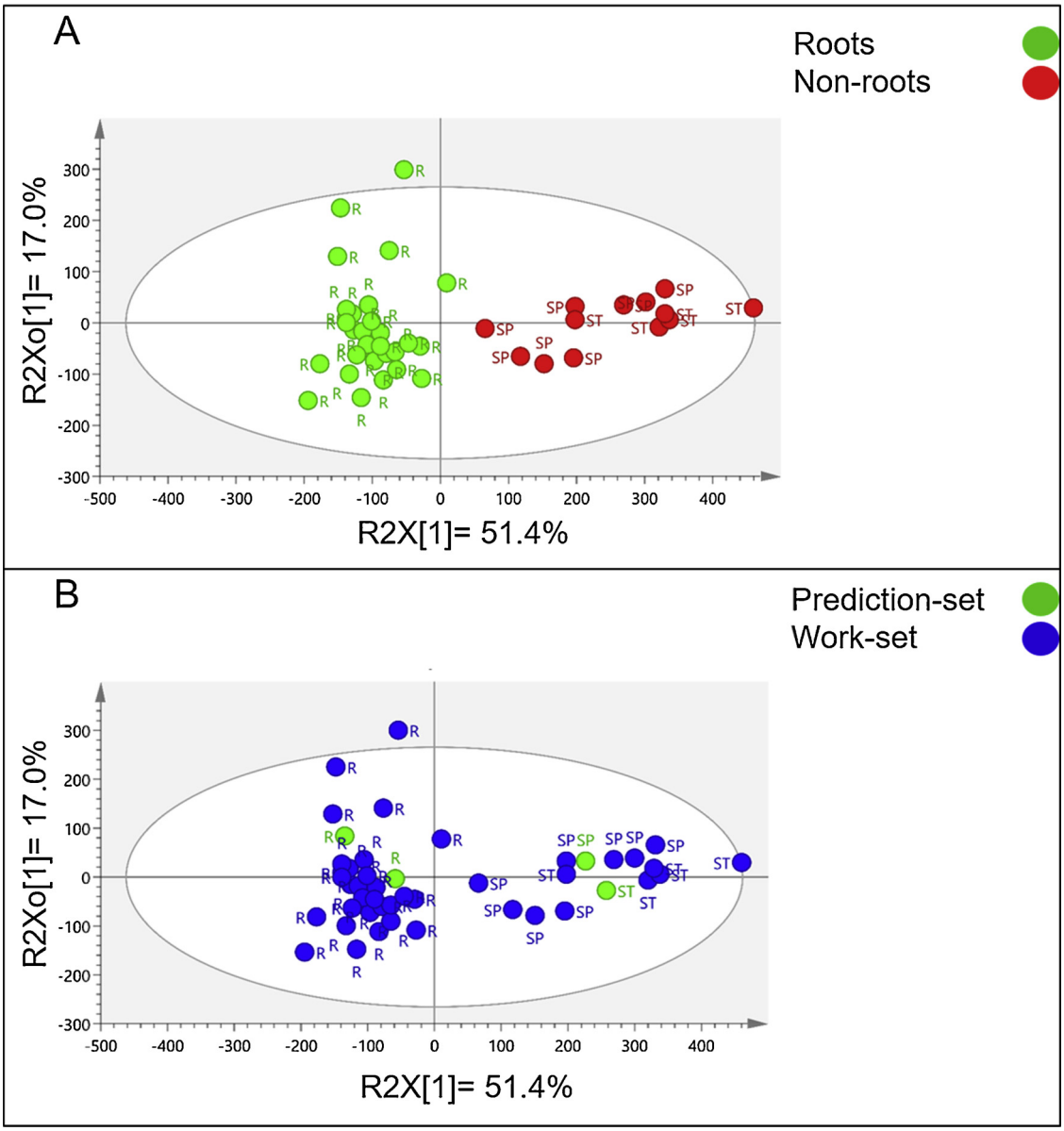

Fig. 2. A) OPLS-DA scores plot derived from UPLC-MS data indicating variables representing two sample classes, namely roots *versus* non-roots and B) Prediction model indicating four external samples predicted correctly by the model.

2.5.2. Chemometric modeling of the data

A partial least squares-discriminant analysis (PLS-DA) model was constructed from the HSI data. Although PLS-DA is merely an alternative form of PLS (partial least squares), it is a supervised method used for membership classification and is used to discriminate new (unknown) samples (Tankeu et al., 2018). To improve the quality and information content of the image, pre-processing (Ctr scaling) and signal correction methods, such as SNV and baseline correction were applied. However, the visual appearance of the image was not satisfactory.

3. Results and discussion

3.1. UPLC-PDA method validation

High performance liquid chromatography is accepted as the reference analytical technique for kavalactone determination in kava (Meissner and Haberlein, 2005). In this study, a new UPLC-PDA method was developed and validated for the determination of the six kavalactones regarded as biomarkers for kava, and for flavokawain quantification. The calibration curves constructed over the range 0.500 – 100 µg/mL were linear as reflected by the coefficients of determination (R^2), which were all above 0.9983 (Table 1). The LODs and LOQs ranged from 0.218-0.437 µg/ml and 0.726–1.46 µg/mL, respectively, indicating that very low concentrations of all the analytes could be determined with confidence. In addition, the highest recovery was 107 % for the 1.25 µg/ml spike of flavokawain B, while the lowest was 94.8 % for the 50.0 µg/ml spike of the same analyte. Since a recovery range of between 80.0 and 120 % is regarded as acceptable (Meissner and Haberlein, 2005), these results are indicative of an accurate method (Table 1). The percentage RSDs reflected the robustness of the method (International Conference on Harmonisation (ICH, 2005). Intra-day precision was conducted over two intervals, with a %RSD ≤ 0.03 obtained for all the compounds, indicating that the degree of scatter was very low. As expected, the inter-day precision was poorer than the intra-day results. However, the RSDs obtained for all of the compounds were below 1 % by Day 2 and below 1.5 % by Day 3 (Table 2). The poorest precision (highest %RSDs) was obtained for dihydrokavain.

The results of this study confirm reports (Siméoni and Lebot, 2002;

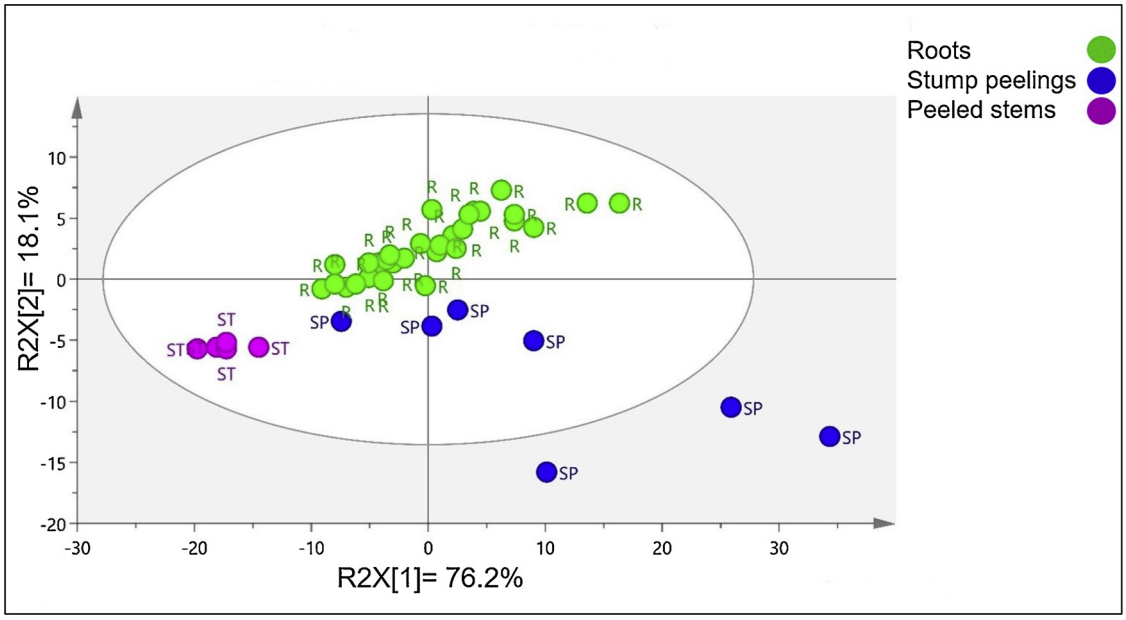

Fig. 3. PCA scores plot derived from UPLC-PDA quantification data indicating a distinction between the roots and non-roots along PC2.

Teschke and Lebot, 2011) indicating that the kavalactone concentrations are higher in the belowground parts than in the aerial parts. Differences in concentrations were particularly prominent when comparing the total averages of the compounds between the roots (45.2 mg/g) and peeled stems (9.22 mg/g) from Tonga (Table 3). Table 4 lists those samples that contained higher kavalactone concentrations in the stump peelings than in the roots.

Roots (65.9 mg/g) from Hawaii contained more kavalactones than samples from the other four countries (Table 3). The Hawaiian roots contained a high concentration of kavain and were characterized by the chemotype 462xxx, which was described by Lebot and Levesque (1996) as the kava chemotype that is fast acting and has a pleasant physiological effect. Hawaiian kava can therefore be considered to induce pleasurable effects. The physiological effects of kavain include emotional stabilization, muscle relaxation and stimulation of creative thoughts (Kretzschmar, 1970). Although the average total kavalactone content of Fijian roots was lower (46.9 mg/g) than that of Hawaiian roots, the chemotype was the same as that of Hawaii, indicating good quality kava.

3.2. Chemometric analysis

3.2.1. Ultra performance liquid chromatography

Raw UPLC-MS data were initially used to create unsupervised PCA models to investigate groupings or clusters within the samples, and to reveal similarities or differences between them (Sandasi et al., 2011). The PCA model with the best model statistics resulted from the Ctr-scaled data and was characterised by three principal components (PCs). This model explained 86.9 % of the variation in the data (R^2X_{cum} = 0.869), while the prediction ability (Q^2X_{cum}) of the model was 66.0 %. The corresponding PCA scores plot (Fig. 1A) indicates two groups separated along PC1, with a variation of 56.2 % between them. The groupings were not based on country or variety, but a clear distinction between the roots and the other plant parts (the stump peelings and the peeled stems) is evident from the plot, confirming that only the plant parts were responsible for the variation. The PCA loadings plot (Fig. 1B) indicates that compounds with *m/z* 229.087, 259.098 and 159.044 are responsible for the distinction between the roots and the other plant parts. An OPLS-DA model was subsequently developed to discriminate the roots (Class 1) and the non-roots (Class 2), which comprised stump peelings and peeled stems. The model statistics were found to be acceptable, with R^2X_{cum} = 0.858, R^2Y_{cum} = 0.842 (indicating the variation between the two classes) and (Q^2X_{cum}) = 0.781 (predictive ability of the model), using two PLS factors (Fig. 2A). The model revealed a 51.4 % variation between the roots and non-roots across PC1 (Fig. 2A). The four external samples were successfully predicted into the model (Fig. 2B), thereby proving its robustness. These results indicate that roots can be distinguished from non-roots using UPLC-MS, even if the samples are diverse and from different regions.

The ability of chemometric models constructed from the UPLC-MS data to distinguish root and non-root samples prompted further chemometric analysis using the concentrations of the marker compounds (six kavalactones and two flavokawains) as the dataset. The model statistics for the PCA model, based on two PCs were as follows: R^2X_{cum} = 0.943 and a Q^2X_{cum} = 0.800. The PCA scores plot is presented in Fig. 3. The corresponding OPLS-DA model yielded an R^2X_{cum} = 0.936, R^2Y_{cum} = 0.632 and Q^2X_{cum} = 0.581, from three PLS factors. The corresponding scores scatter plot and S-plot are presented in Fig. 4A and B.

The three compounds (*m/z* 229.087, 259.098 and 159.044) at the extreme ends of the S-plot (Fig. 4B) can be regarded as those that play the biggest role in the distinction between the roots and non-roots. These were identified as kavain (229.087), methysticin (isomer 159.044) and yangonin (259.098), after comparing the retention times and UPLC-MS spectra with those of the standards. These compounds were consistent with those identified through the loadings plot of the PCA model derived fom the UPLC-MS data. A high concentration of kavain is characteristic of the roots, particularly in *Noble* cultivars (Lebot and Levesque, 1996). In contrast, the compound dihydromethysticin was associated with the non-roots (Fig. 4B) and, according to the quantitative results, the concentrations of dihydromethysticin were higher in these parts (Table 4). According to kava quality regulations in the South Pacific, kava must not be prepared from stump peelings.

3.2.2. Near-infrared spectroscopy

The best PCA model (six PCs) in terms of model statistics was constructed from Ctr-scaled and MSC-filtered data (R^2X_{cum} = 0.996, Q^2_{cum} = 0.993). Variation between the roots, the stump peelings and peeled stems was observed across PC1 plotted against PC5 (Fig. 5A). The root samples scattered along the positive PC5, while those representing stump peelings and peeled stems were distributed

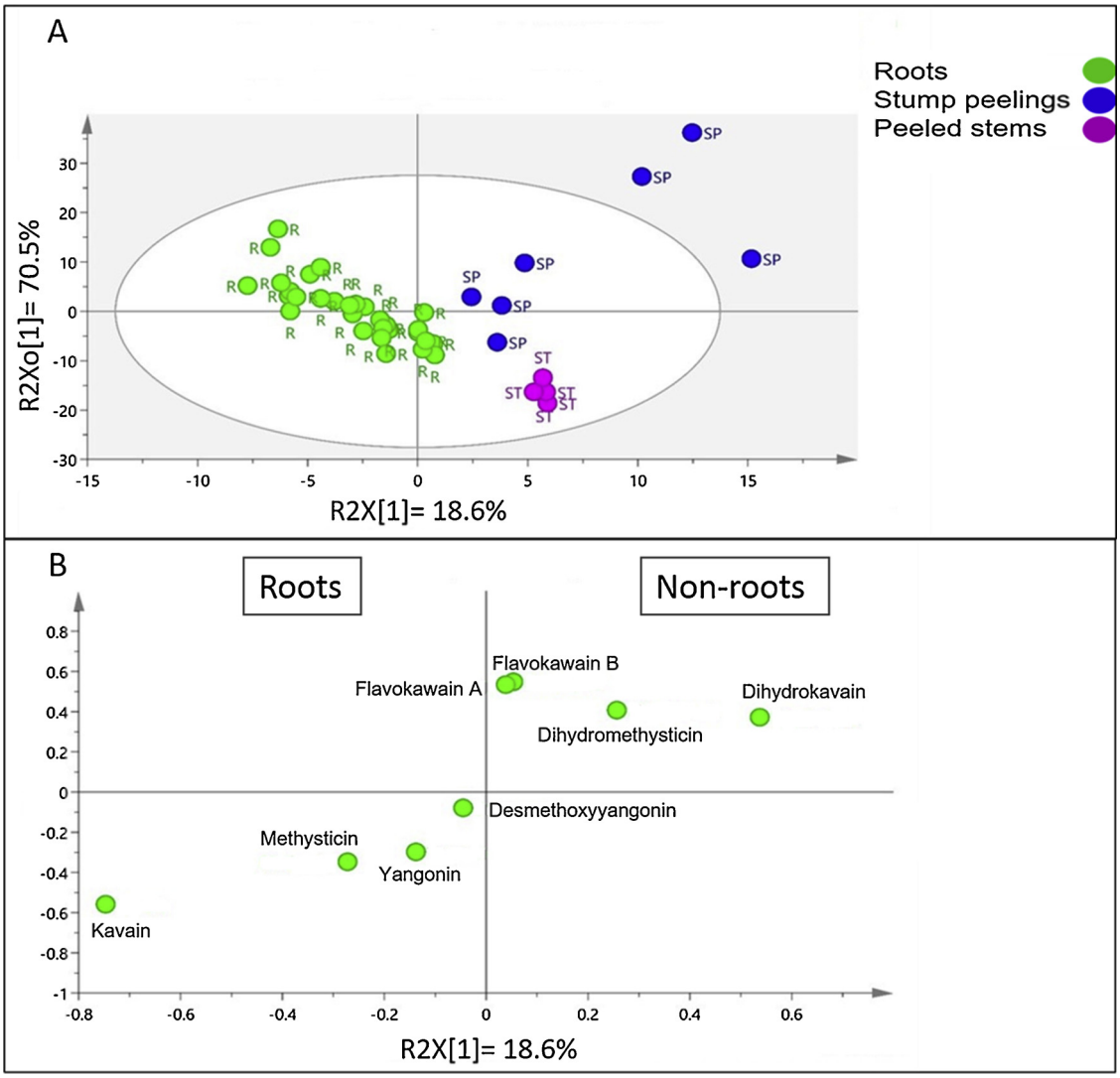

Fig. 4. A) OPLS-DA scores plot derived from UPLS-DA quantification data and B) corresponding S-plot derived from the concentrations of the kavalactones and flavokawains as determined in kava extracts using UPLC-PDA.

throughout the negative PC5. The lack of groupings indicate that the peeled stems and stump peelings have a similar chemistry. The corresponding OPLS-DA model constructed to discriminate roots from non-roots, also yielded acceptable statistics (R^2X_{cum} = 0.992, Q^2X_{Cum} = 0.622, R^2Y_{cum} = 0.724) and was characterised by four PLS factors (Fig. 5B). However, there was only a 2.5 % variation between the roots and non-roots. Despite this shortcoming, the model classified all of the external samples correctly (Fig. 5C).

3.2.3. Mid-infrared spectroscopy

The Par-scaled and MSC-filtered NIR data yielded the model (13 PCs) with the best statistics (R^2X_{cum} = 0.996) (Fig. 6A). The root samples were separated from the other plant parts (stump peelings and peeled stems) by PC2, with root samples scattered in the positive PC2. Moreover, the stump peelings and peeled stems again clustered together, which is indicative of a similar chemistry between the two plant parts. The PCA model was used to develop an OPLS-DA model, based on the two classes assigned (roots or non-roots). The statistics of the model were good (R^2X_{cum} = 0.947, Q^2X_{cum} = 0.589 and R^2Y_{cum} = 0.764) (Fig. 6B), with a 9.87 % variation indicated between the roots and non-roots. The same external work-set as before were predicted by the model, to evaluate the robustness of the model (Fig. 6C), with all samples correctly predicted as roots or non-roots.

The results of both NIR and MIR indicate that vibrational spectroscopy can be applied to discriminate roots and non-root samples. However, the MIR results were more reliable, as a larger degree of variation between the plant parts was achieved.

3.2.4. Hyperspectral imaging

A PCA model was developed from an HSI image (hypercubes) of different plant parts (roots, peeled stems and stump peelings). The quality of the hypercube was improved through Ctr-scaling of the model, followed by MSC and Baseline correction to filter the data. The resulting scores plot (Fig. 7A) and scores contour image (Fig. 7B) were subjected to the trend tool (MIA toolbox, MATLAB) to improve separation and chemical variation between the different plant parts used.

To ensure that only relevant spatial and spectral information was used in the model, wavenumbers that did not contribute to the discrimination of the different plant parts were identified and discarded. Finally, a smaller dataset (Fig. 8A and B), comprising 99 (region 6 nm–105 nm) out of the 256 variables initially obtained from the instrument, was retained. Thereby the initial dataset (920–2514 cm-1) was reduced to a smaller dataset (751.77–1574.2 cm-1) containing only the distinguishing information. After reduction of the dataset, the resulting PCA scores scatter plot and scores contour image (Fig. 9A and B) indicated a better chemical variation compared to the initial plots using

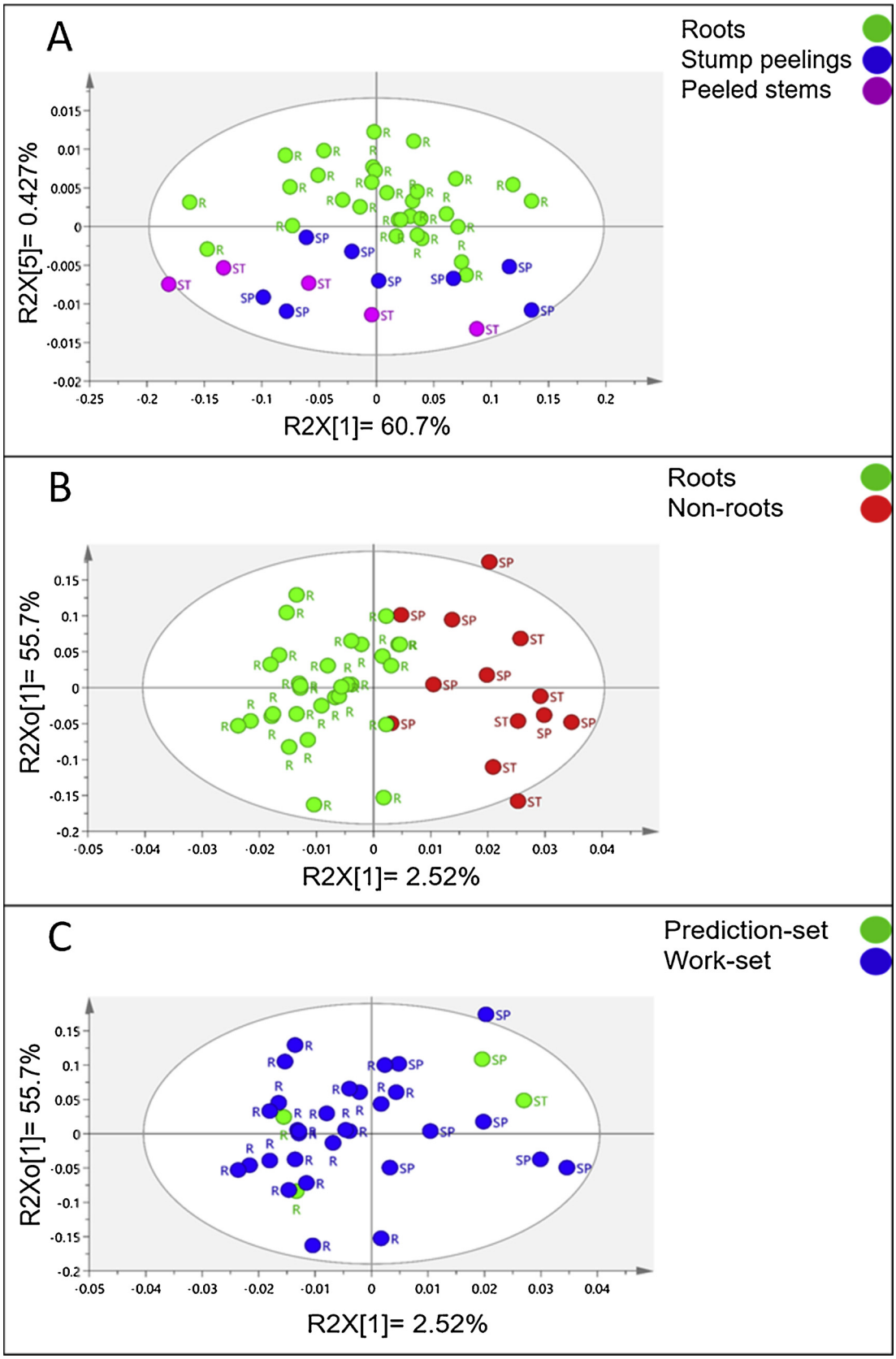

Fig. 5. A) PCA scores plot, B) an OPLS-DA scores plot derived from NIR data, indicating separation between roots and non-roots and C) B) external samples correctly predicted by the model.

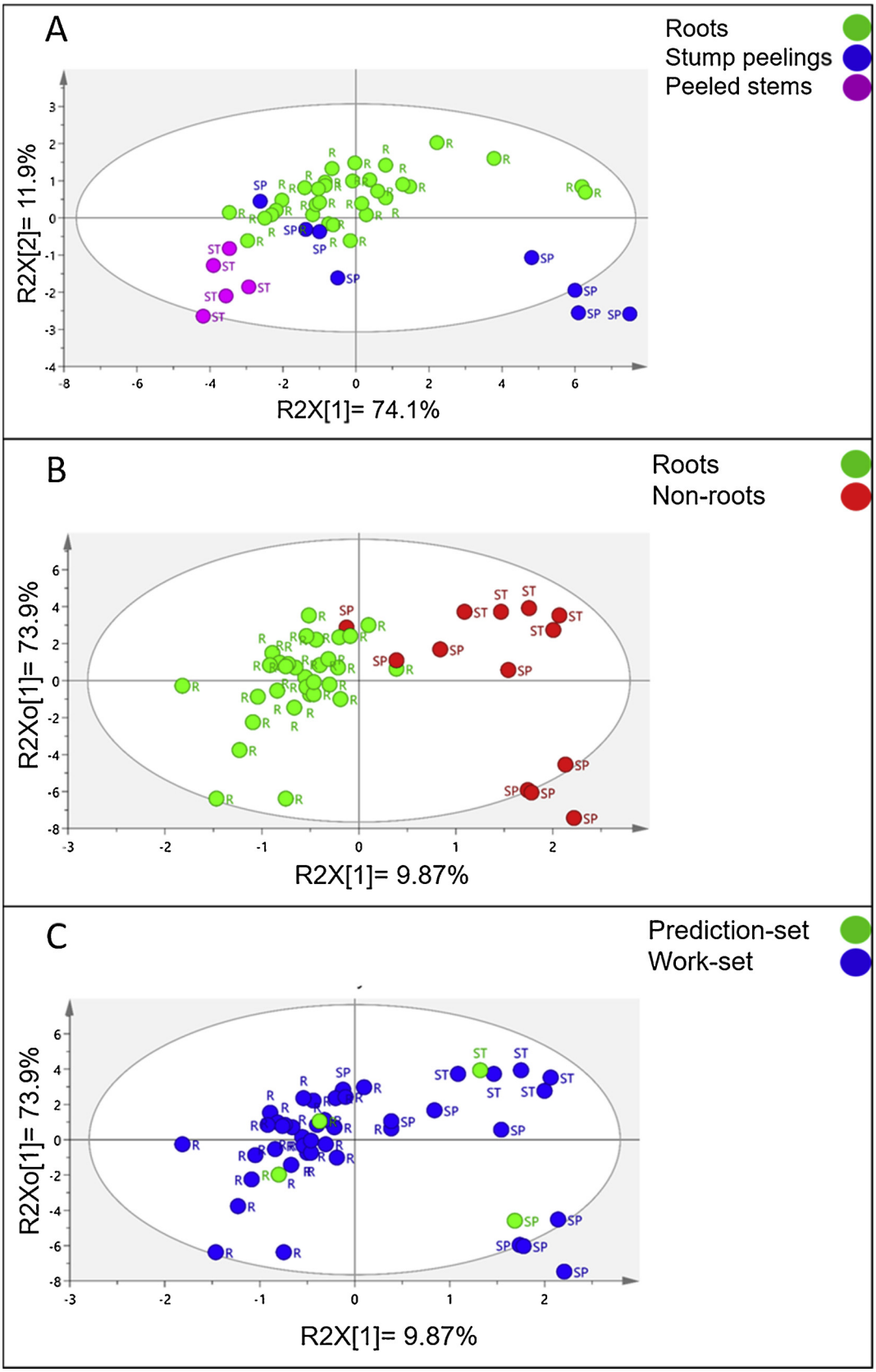

Fig. 6. A) PCA scores plot, B) OPLS-DA scores derived from MIR data, indicating separation between roots and non-roots and C) external samples correctly predicted by the model.

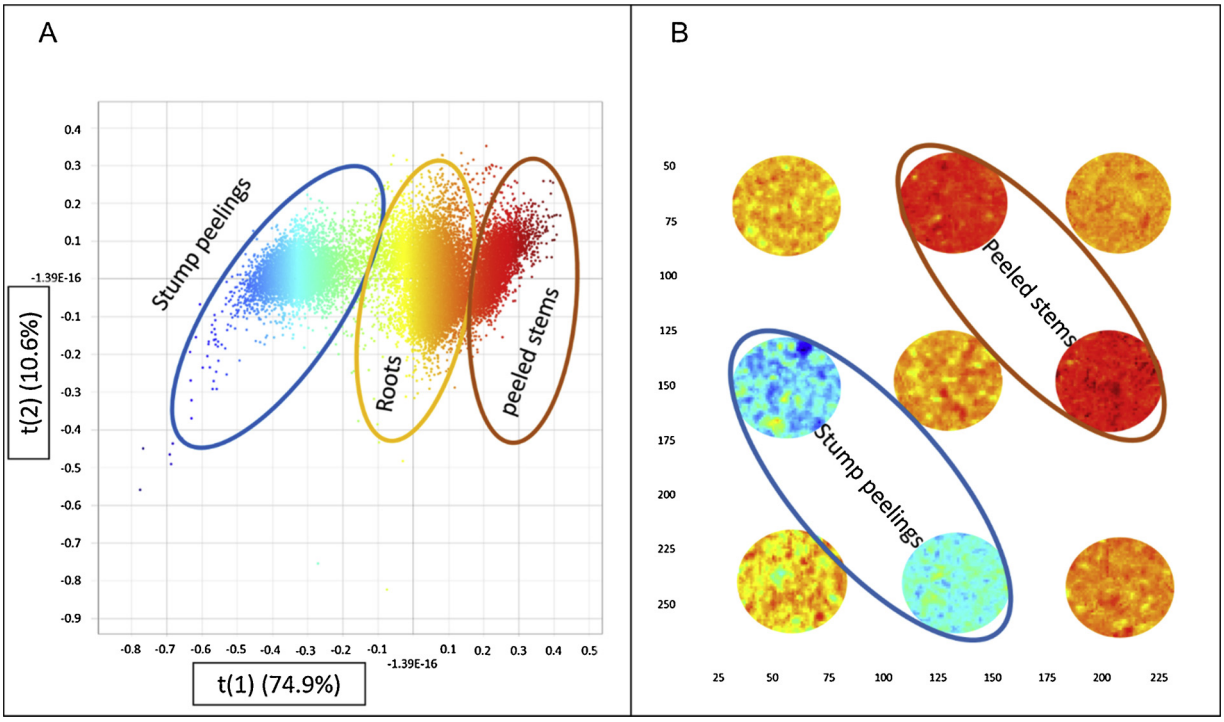

Fig. 7. A) PCA scores plot derived from HSI data after application of scaling and spectral filters and B) a contour image indicating clear distinction between the sample-types.

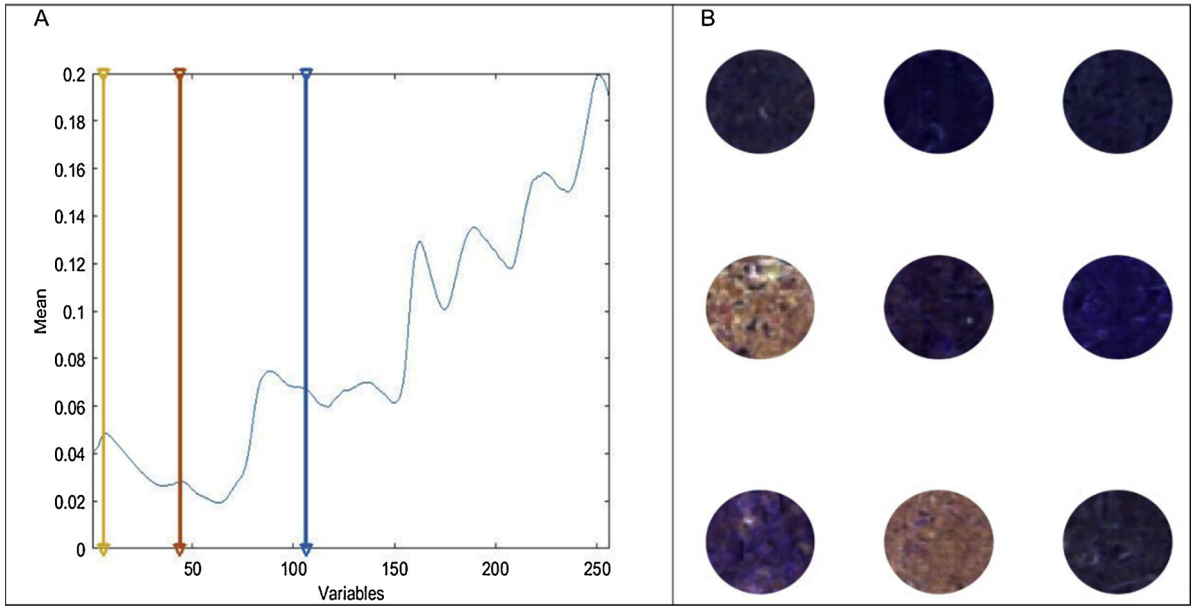

Fig. 8. A) Interactive investigation of different waveregions and B) scores contour image obtained after applying the MATLAB R2018 trend tool.

the entire spectral range.

Finally, a PLS-DA model was created using the two classes (roots and non-roots) to discriminate the roots from the stump peelings and peeled stems (Fig. 10). The obtained model was tested for robustness by predicting external samples (three roots, two stump peelings and one peeled stem) into the model, all of which were predicted with a high accuracy. The most accurate root prediction was 91.1 %, while that of non-roots was 92.1 % (Fig. 11)

4. Interpretation of the chemometric results

Several studies, including those by Siméoni and Lebot (2002) and Lasme et al. (2008), indicated that the kavalactone content of the roots is higher than that of the aerial parts of the shrub. Kava standards in the South Pacific countries of origin and the Draft Regional Standard for Codex Alimentarius define that kava rootstock (rhizome) must be peeled. The stump peelings are usually discarded. However, in this study there were several cases where the stump peelings of some cultivars contained more kavalactones than the roots (Table 4). This implies that using kavalactone content as a marker to discriminate plant parts, may not always work and would not allow determining whether the rootstock was properly peeled. In all these cases, the flavokawain content (flavokawain A and flavokawain B) was consistently higher in the stump peelings than in the roots. Therefore, including the

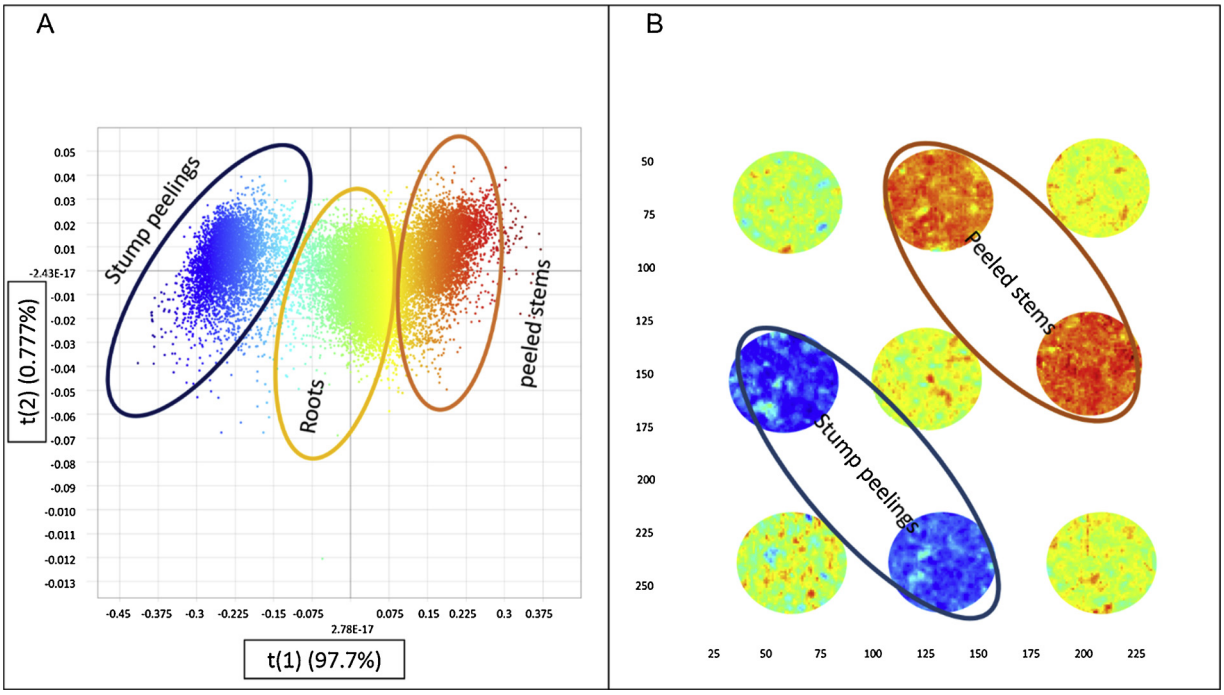

Fig. 9. A) PCA scores plot derived from HSI data after the spectral wave was reduced and B) scores contour image indicating differences between the sample types.

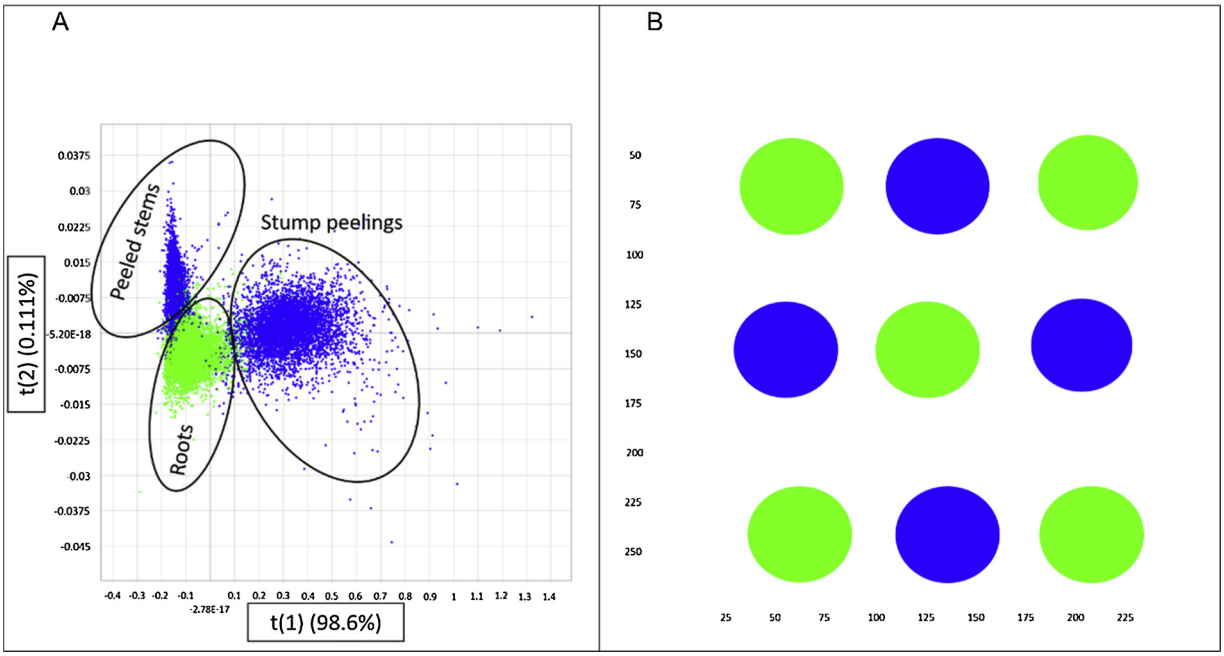

Fig. 10. A) PLS-DA scatter plot and B) contour image, indicating the root (green) and non-root (blue) samples.

quantitative results of flavokawain A and flavokawain B may improve quality control, which corresponds to the findings of an HPTLC analysis of Lebot et al. (2019). However, caution should be taken before comparing results with those reported by Siméoni and Lebot (2002), Lasme et al. (2008) and Lebot et al. (2019), since they investigated only samples from Vanuatu. In the current study, samples from Fiji, Hawaii and Samoa were included, thereby increasing the variability. Several studies (Gautz et al., 2006; Lasme et al., 2008; Lebot et al., 2014; Lebot and Legendre, 2016) have proposed reliable and robust methods for kava quality control. However, the limitation of these studies is that the sample set originated from only one country (Vanuatu), with the exception of the study of Gautz et al. (2006), who analysed samples from the South Pacific (origins and number not mentioned) and Hawaii. The standard of quality proposed by the Amended Vanuatu Kava Act No. 6 of 2015 that stipulates the use of the *Noble* cultivar, locally and for export, with plant parts restricted to the roots and peeled rhizomes of the shrub, pose a challenge in quality control. There is no regulation dictating the form of kava for export, and there is a lack of appropriate quality control methods to determine if roots, peeled rhizome and/or powder originate from kava. Kava is easier to handle when powdered, and powders have been utilized in most studies. It is therefore important to develop inexpensive and rapid quality tests to determine the parts of origin for powdered kava raw materials. Gautz et al. (2006) succeeded in determining the total kavalactone concentration using

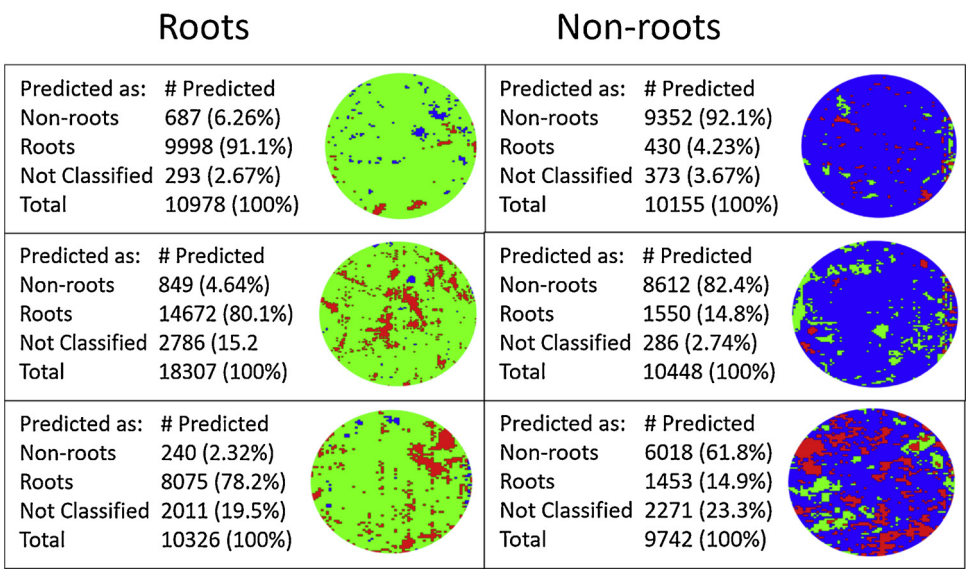

Fig. 11. External prediction results for three roots (green) and three non-roots (blue) using the PLS-DA model constructed from HSI data.

NIR spectroscopy, producing results comparable to those of the HPLC method. However, total kavalactone content does not reflect the cultivar, plant part, or age of the cultivation. Flavokawains were used to identify *Noble* cultivars from Vanuatu in the studies by Lebot et al. (2014/2019).

The chemometric methods developed in this study seek to address the issue of plant part authentication in terms of discriminating the roots from other parts of the shrub. Since the roots are the preferred plant part for local use and export, this aim has merit. The chemistry of the roots was found to be clearly different from that of the stump peelings and peeled stems, when both chromatographic and various spectroscopic techniques were applied. The spectroscopy results were in agreement with those of UPLC-MS/PDA. The main advantage of using HSI in this study is that the analytical technique has the potential to be automated and is capable of continuously scanning multiple samples.

5. Conclusions

The determination of the kavalactones and flavokawains confirmed prior reports that the concentrations of the kavalactones are higher in the underground parts of kava than in the aerial parts. However, it was found that for some varieties, the stump peelings did contain more kavalactones than the roots. This is concerning, since the analytical results revealed much higher concentrations of flavokawains in the stump peelings than in the roots. Since there are no guidelines on the safety of the flavokawains, it is advisable that stump or stem peelings not be used. The development of methods to discriminate the roots from other kava plant parts is important, because it is impossible to determine the plant part of origin once kava has been ground into powder. In this study, a new UPLC-PDA method for the simultaneous determination of six kavalactones and two flavokawains was developed and validated. Three quality control methods based on vibrational spectroscopy are proposed for discriminating roots from non-roots. The application of NIR and MIR spectroscopy and HSI, in combination with chemometric analysis, produced reproducible results that were confirmed by the use of UPLC-MS.

As the method is sensitive to differences in FKB contents, it might also be applied to kava nobility testing at the point of export. The samples used in this study were all *Noble* kava varieties, therefore a continuation of the study by including non-noble varieties is expected to give results of potentially high importance for kava quality testing with an easy to use, robust and reliable method at the points of exportation in the countries of the South Pacific.

Declaration of Competing Interest

None.

Acknowledgements

The authors express their gratitude to the National Research Foundation (NRF) of South Africa and the South African Medical Research Council for funding.

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