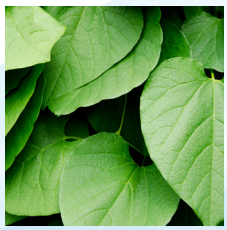
HORIBA

Life Science

Quantitative Chemometric Modeling of Kavalactones and Flavokavains in Kava Root with A-TEEM™ Fluorescence Spectroscopy



Application Note
Agricultural Sciences
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1. Flora Research Laboratories, 2. HORIBA Scientific,
3. University of Mississippi for supplying of reference compounds

Abstract

Kava (*Piper methysticum*) is a shrub in the pepper family that is native to the islands of the South Pacific. The root is traditionally consumed as a beverage for recreational and therapeutic purposes, and it has a reputation as a sedative, anxiolytic, and promoter of sociability. It has gained popularity outside of its endemic region and is widely available. Because different pharmacological effects are linked to different varieties of kava, and because variety can be linked to a profile of the constituents kavalactones and flavokavains, measurement of these components promotes safe and effective use of the plant. In this application note, a method for accurately predicting the amounts of major constituents of kava root from spectroscopic absorbance and fluorescence measurements made with the HORIBA Aqualog® and the A-TEEM technique is presented. A partial least-squares regression chemometric model is built using a set of kava samples with known chemistry, and refinement of the model and appropriate scope for its application are discussed.

Introduction

There are over a hundred cultivars and chemotypes of Kava (*Piper methysticum*) grown throughout Polynesia, Micronesia, and Melanesia that that contain varying amounts of kava's main pharmacologically active constituents. These include six kavalactones and three chalconoids known as flavokavains. Their structures are shown in Figures 1 and 2.

Quantification of these constituents is important for monitoring the quality of kava sold for consumption and for the manufacturing of standardized extracts. Kavalactones are lipophilic molecules present in the root resin and may account for a significant percentage (3 to 20%) by weight of dry material (Rowe 2011). Flavokavains have demonstrated anti-cancer effects in vitro and seem to potentiate the kavalactones (Abu 2013). Of these nine constituents, flavokavain C is present is the smallest amounts, and is often below detection limits of HPLC methods. As with cannabis, different cultivars of kava are associated with different physiological effects. For example, varieties of kava from Vanuatu known as "noble" kava have a reputation for reliably producing pleasant tranquility and are the only of the island's four major variety types approved for export. Another Vanuatu variety type known as "two day" kava appears to cause more instances of adverse effects like nausea and headache (sometimes lasting multiple days). Analysis of the two strain types shows that "noble" kava chemistry is dominated by kavain, whereas "two day" contains more dihydrokavain

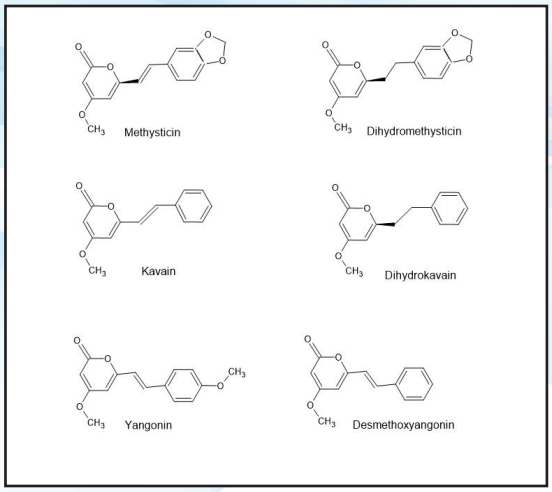

Figure 1: Kavalactones found in kava root

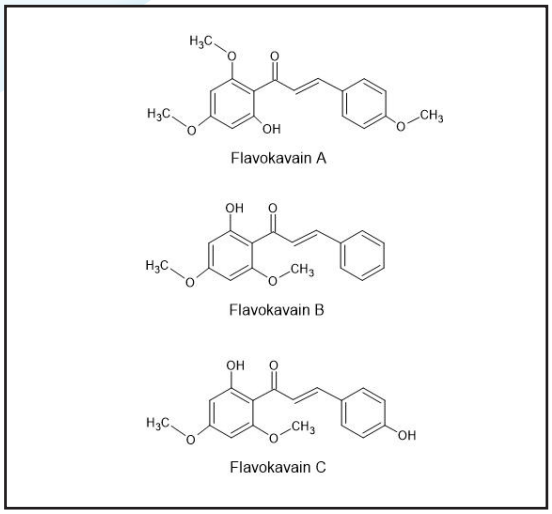

Figure 2: Flavokavains found in kava root

and dihydromethysticin (Bian 2020). Whether these last two chemicals cause the ill effects or are merely correlated with unknown factors is unclear, but the kavalactone and flavokavain fingerprint of a root can indicate whether one has the desired strain likely to produce the desired outcome.

HPLC is the most common technique for analyzing kavalactones and flavokavains. Containing resonant ring structures, these molecules have strong native fluorescence, which suggests that simpler, cheaper, more sensitive, and more environmentally-friendly fluorescence spectroscopy could be substituted for chromatography for their quantitation. A-TEEM Spectroscopy is a robust, fast, and very sensitive technique that combines simultaneous measurement of absorbance, transmission (A-T), and fluorescence excitation emission matrix (EEM). A three dimensional A-TEEM data set allows for automatic correction of the inner filter effect, extending the linear range of fluorescence spectra, thus providing undistorted true molecular fingerprints.

A-TEEM data, combined with HPLC assay results, can be used to build a quantitative predictive chemometric model for kava constituents. A robust model that covers the variable space will be based on numerous diverse, well-characterized examples. Partial Least Squares (PLS) regression is a good model choice when a large set of correlated independent variables (such as the EEM and absorbance data) are used to predict a much smaller set of dependent variables (the quantities of kavalactones and flavokavains). PLS regression finds a small set of latent variables that can explain the data.

Flora Research Laboratories, a third-party testing lab in Oregon, US, routinely analyzes kavalactones and flavokavains in dry kava root by HPLC. Using HORIBA's Aqualog® UV-800 Spectrophotometer and this abundant trove of data, the group constructed a model that reliably predicts six kavalactone constituents from rapid A-TEEM spectroscopy measurements. They also investigated predicting flavokavains A and B. Because flavokavain C is often below the limit of quantitation for HPLC, it was excluded from this study.

Experimental Procedure

Dry kava root samples that had been assayed by HPLC were prepared with the same extraction that had been used for the chromatographic analysis of 200 mg in 50 mL of 70% LCMS-grade methanol in water. They were diluted by an additional factor of 80, determined by observing the optical density in the absorbance measurement, and aiming for a maximum between 0.25 and 0.75 for the majority of samples. The samples were placed in quartz cuvettes with 1 cm path length. The cuvette temperature was maintained at 20°C with circulating chilled water. Aqualog® A-TEEM measurements were made with a 230 – 600 nm excitation range with a 5 nm increment, a 246 – 823 nm emission range with a 4.6 nm increment, a 0.4 second integration time, and with the CCD set to medium gain. Each measurement took about one minute.

The Aqualog® software (V4.3) provided data conditioning including blank subtraction, interpolation, masking of Rayleigh and Raman peaks, and normalization to the water Raman peak area to account for inter-day variability in the spectrophotometer lamp intensity. A total of 54 unique samples were measured. Typical kava A-TEEM and absorption measurements are shown in Figures 3 and 4, respectively.

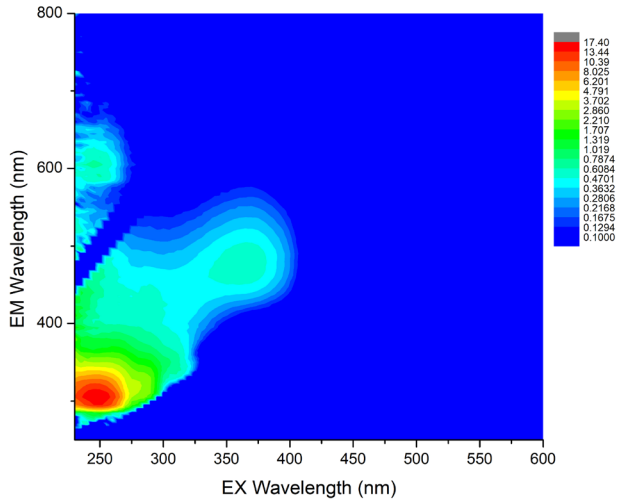

Figure 3: Typical kava A-TEEM

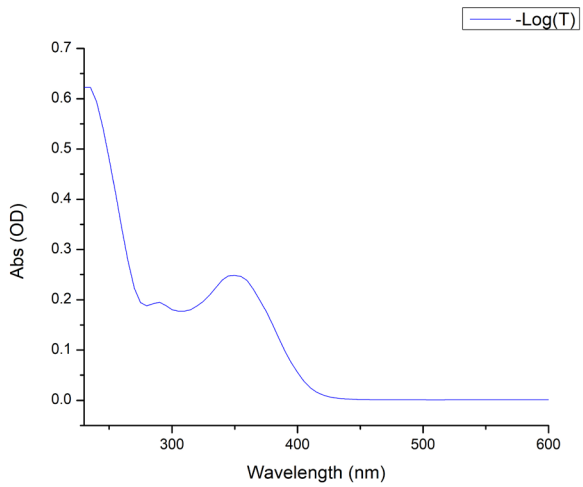

Figure 4: Typical kava absorption spectrum

Chemometric Model

The model used was a partial least-squares (PLS) regression. It was built in Solo software (version 9.0) from Eigenvector Research, Inc. For each sample the EEM data was first unfolded into a series of 2-dimensional emission spectra, one for each of the 75 excitation wavelengths. These were concatenated into one long vector, to the front of which the absorption spectrum for that sample was appended. The 54 samples were loaded into the independent "X" matrix in a PLS-Regression model in the software. The dependent "Y" matrix was constructed from the µg/g of each of the 9 constituents as determined by the HPLC measurements. Because the samples had been weighed out and prepared a second time for the A-TEEM measurements, a correction factor was applied to each for the exact concentration used. Both the X and Y matrices were mean-centered. Cross-validation was achieved with a Venetian-blind technique with 10 splits and a "blind" thickness of 1.

Individual models were built for each of the nine constituents. To identify outlier samples unsuitable for inclusion in the final models, Hotelling T^2 distances versus Q-residuals were plotted for each (see example in Figure 5). The samples with either high residuals, such as the one represented by the dark green square in the upper left in Figure 5, or great distance from the mean, or both, such as the light green square in the upper right quadrant, are poorly explained by the model. They may represent measurement error or simply be unusual, atypical kavas. The model was refined by iteratively removing such samples, building a model with the remainder, and re-assessing the set for outliers. Final models for the kavalactones and flavokavains had between 45 and 49 samples out of the initial 54.

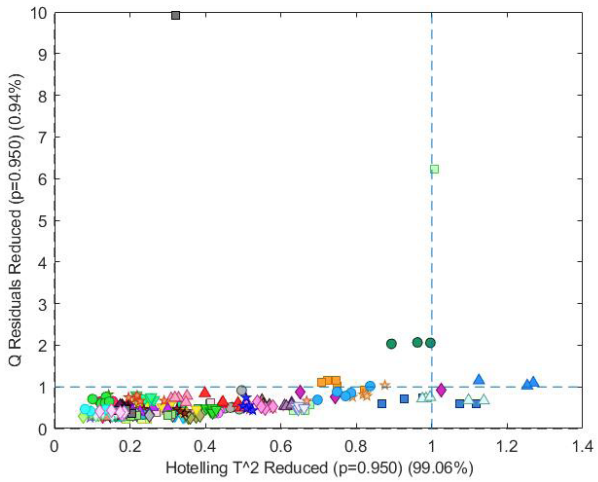

Figure 5: Hotelling T^2 vs. Q Residuals for initial methysticin model showing outlier samples.

Observing the plot of the root-means-square errors of calibration and cross-validation (RMSEC and RMSECV, respectively) versus LV number (see Figure 6), the number of latent variables that balanced over- and under-fitting of the data was chosen for each of the nine kava constituents. An example is shown in Figure 6.

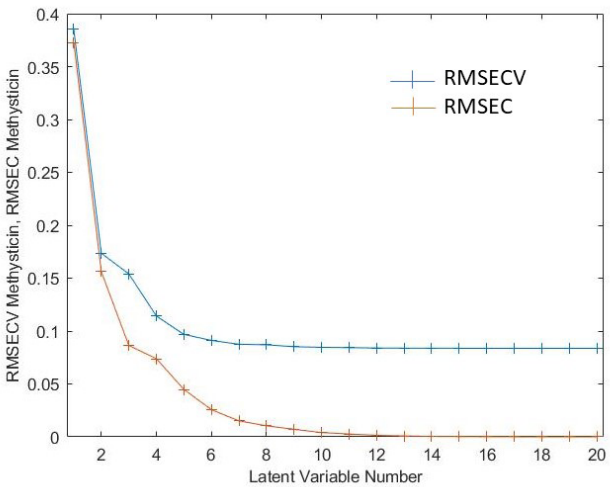

Figure 6: Latent variable number vs. RMSEC and RMSECV for methysticin model. LV number of 6 was selected.

A plot showing HPLC-measured versus cross-validation predicted amounts of methysticin made with the refined model is shown in Figure 7. The coefficient of determination of R^2 of calibration is 0.9934, and the R^2 of cross-validation is 0.9590.

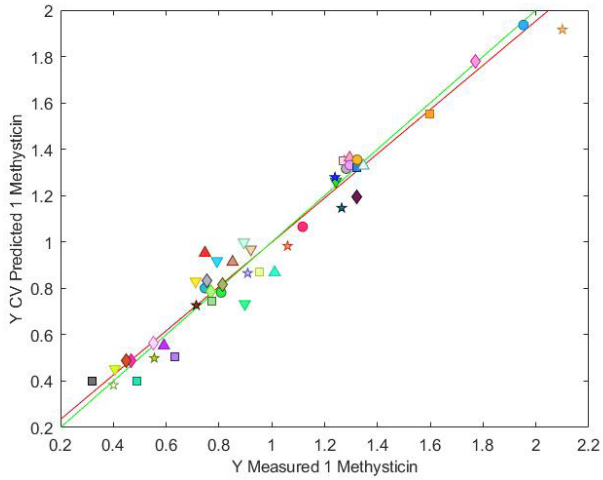

Figure 7: Measured vs. CV predicted methysticin for refined model.

Results and Discussion

The models produced for each constituent and their performance are shown in Table 1.

Constituent	Number Samples	LV Number	R^2 Calibration	R^2 Validation
Methysticin	45	6	0.9934	0.9590
Dihydromethysticin	49	5	0.9921	0.9539
Kavain	46	6	0.9989	0.9790
Dihydrokavain	45	6	0.9968	0.9360
Yangonin	45	6	0.9969	0.9457
Desmethoxyyangonin	45	5	0.9897	0.9457
Flavfokavain A	45	5	0.9830	0.8732
Flavfokavain B	45	5	0.9825	0.8563

Table 1: Models and performance for kava constituents

The difference between the calibration and cross-validation R^2 values is an indicator of a PLS model's fitness for new samples not in the training set, but within the covered variable space. The table shows that the models for the kavalactones will be good predictors, but that the flavokavains would benefit from additional training data. More samples would improve the models, as would spiked samples. When including spiked samples, one must have a precise knowledge of the spike composition in order to actually improve chemometric models with this very sensitive and non-separative A-TEEM technique. For example, a 98% pure flavokavain A standard with trace amounts of kavalactones and other flavokavains will worsen the model's performance unless the quantities of those other constituents are also accurately known and included in the dependent variable matrix, as their fluorescence will be detected by the Aqualog.

Proper use of a predictive chemometric model as a replacement for chromatographic analysis requires determining whether a particular new sample falls within the variable space of the existing model. This is accomplished with the same procedure for identifying outlier samples that was discussed in the Chemometric Model section. If a new kava sample would qualify as an outlier for the existing model, it would be a better candidate for a different analytical technique, such as HPLC. Another consideration is the need to maintain a model that is appropriate to the samples needing testing. That is, if the types and chemistry of kava on the market change, the models must be expanded and updated as well, while maintaining quality standards.

Conclusion

Quantifying kava's active principles is important for ensuring high quality and safety for consumers wishing to enjoy its recreational or therapeutic benefits. Predictive chemometric models combined with HORIBA Aqualog® A-TEEM spectroscopy offers a viable alternative to HPLC analysis for the kavalactones in kava root. The advantages of this switch include faster, more cost-effective, and potentially "greener" testing. A-TEEM gathers abundant data quickly and takes advantage of the high sensitivity of fluorescence-based methods, and is a technique likely to be adopted in many similar fields in the future.

References

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