JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2006,** *54,* 6147−6152 **6147**

Determination of Kavalactones in Dried Kava (*Piper methysticum*) Powder Using Near-Infrared Reflectance Spectroscopy and Partial Least-Squares Regression

LOREN D. GAUTZ,*,† PAKIELI KAUFUSI,† MEL C. JACKSON,‡ HARRY C. BITTENBENDER,† AND CHUNG-SHIH TANG†

Department of Molecular Biosciences & Bioengineering, University of Hawaii at Manoa, Honolulu, Hawaii 96822

Kava (*Piper methysticum* Forst F.), or àwa in the Hawaiian language, has been used for thousands of years by the people of the South Pacific Islands, in particular Fiji, Vanuatu, Tonga, and Samoa, for social and ceremonial occasions. Kava has the unique ability to promote a state of relaxation without the loss of mental alertness. Kava recently became part of the herbal pharmacopoeia throughout the United States and Europe because of its anxiolytic properties. The active compounds are collectively called kavalactones (or kava pyrones). The need for a less time-consuming and costly method to determine the concentration of kavalactones in dried kava is urgent. The combination of near-infrared reflectance spectroscopy (NIRS) and partial least-squares (PLS) methods has been found to be a convenient, versatile, and rapid analytical tool for determination of kavalactones in dried kava powder. Calibration equations were developed based on the analyses of 110 samples with variable physical and chemical properties collected over time from Hawaii kava growers and validated by analyses of a set of 12 samples with unknown kavalactones concentration. All six major kavalactones and the total kavalactones were measured using NIRS with accuracy acceptable for commercial use. The NIRS measurements are reproducible and have a repeatability on a par with HPLC methods.

KEYWORDS: Kavalactones; kava; *Piper methysticum*; near-infrared; reflectance; spectroscopy; partial least-squares

INTRODUCTION

The underground organs of *Piper methysticum* Forst. f. have been used to prepare a beverage in the Tropical Pacific for millennia (*1*). This plant, fresh and dry organs, beverage, and any other preparation from the organs are called kava in common American usage although other names are used.

The kava beverage and other preparations are known to be anxiolytic. In recent times preparations have been used as medicine or as nutraceuticals in the treatment of anxiety, restlessness, and insomnia (*2*). Kava has the unique ability to bring about a state of relaxation without loss of mental alertness (*3*, *4*).

The safety of the beverage is well established (*1*). There have not been any cases of toxicity or long-lasting debilitative side effects with the beverage. The beverage is known to cause dry, scaly skin with extensive use, but this condition is not debilitative; it only occurs with exorbitant use over a period of months and stops with cessation of drinking the kava beverage (*2*). The ban of kava by various European countries was due to alleged hepatotoxicity of tablets of extracted and concentrated kavalactones. It is widely believed the cause of this toxicity was in the process (*2*) and/or plant tissues (*5*) used to prepare the tablets (*6*). Many of the bans have been lifted or are in the process to be lifted soon (*7*).

The market for kava has contracted in the face of the ban by European countries; however, an international market still exists. Furthermore, with the expected reopening of European markets, orderly trade is greatly desired.

For orderly trade there must be impartial establishment of the quality of the kava being exchanged. The closer to the point of origin the quality is established, the more efficient the market can be. Quality for kava is generally regarded as the kavalactone profile and total kavalactones content. Quality is also given by the chemotype. The relative concentrations of each of the six major kavalactones in roots give a chemical profile. The relative concentrations are also commonly expressed in qualitative form called a chemotype. The six major kavalactones have been assigned a number as follows: (1) desmethoxyyangonin, (2) dihydrokavain, (3) yangonin, (4) kavain, (5) dihydromethysticin, and (6) methysticin. The six digits arranged from highest to lowest concentration of the corresponding kavalactone give the kava chemotype (*8*).

* To whom correspondence should be addressed. Phone: 808 956-6876. Fax: 808 956-9269. E-mail: lgautz@hawaii.edu.
† University of Hawaii at Manoa.
‡ Hawaii Agriculture Research Center.

10.1021/jf060964v CCC: $33.50
Published on Web 08/02/2006

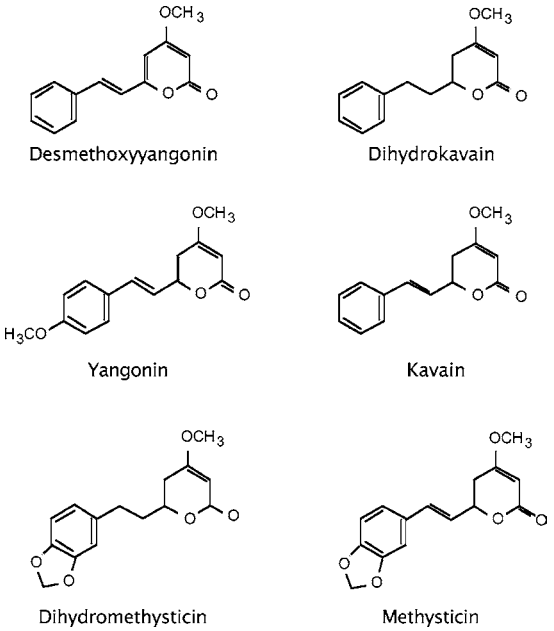

Figure 1. Structure of six major kavalactones.

The chemistry of kavalactones in kava has been well studied, and methods are available for qualitative and quantitative analysis. For example, HPLC/MS analysis (*9*) showed 18 identifiable kavalactones and three chalcones. Another recent report (*10*) showed that the enantiomeric separation of kavalactones could be achieved using chiral active HPLC columns. For the quantitation of the six major kavalactones (**Figure 1**), a reverse-phase HPLC method has been reported (*11*). Capillary GC analysis (*12*) offers another possible approach for kavalactone analysis. These chromatography methods are all relatively expensive and time-consuming. Considerable resources, e.g. solvent, physical facility, and time, are used just to prepare the sample for analysis.

NIRS has been increasingly used for the analysis of natural products in solid samples (*13*). This method can perform reliable quantitation in small amounts of samples, often without time-consuming sample preparation procedures, if the analyte exceeds 0.1% of the composition. NIRS has been used to quantify kavain and total kavalactones in purchased dry kava extract samples (*14*) but was not used on dried kava root, the basic commodity of trade, and is therefore of limited utility in international raw materials markets.

NIR spectra contain harmonics of the fundamental absorption bands of the analyte. The absorption peaks are broad and often overlap, making single wavelength calibrations impossible. A useful multivariate calibration tool is available in the form of PLS. PLS is a form of principal component analysis that makes use of the information in the full NIR spectrum (400−2500 nm) and the established analyte value associated with the spectrum. The calibration equation can be applied to unknown samples once the equation is established by PLS from samples where the analyte is determined by another acceptable method.

This study newly demonstrates that NIRS offers an efficient and economical method for determining kavalactone profiles and chemotype by determination of the concentration of all six major kavalactones as well as the total kavalactone. Furthermore, the protocol developed determines content of total kavalactone and specific kavalactones from dry powdered underground organs—root and caudex—without extraction.

The current HPLC method of analysis for determining kavalactone content and chemotype are a significant expense to the small farmer, costing as much as $350 per analysis in some laboratories. In addition, the majority of kava root buyers require an indication of the kavalactone content of the root before they will make a purchase. The fast, accurate means of quantifying all six individual kavalactones by NIRS puts this type of certification within the financial reach of growers. Scientists studying kava also desire such a methodology.

Another benefit of using NIRS for routine determination of kavalactones concentrations is the elimination of solvents in the sample preparation. NIRS can be applied to the raw product in most cases with only limited mechanical treatment.

MATERIALS AND METHODS

Samples of underground organs were taken from kava plants grown under various cultural practices. Most of the kava was grown at the University of Hawaii Magoon Research Facility in Manoa Valley, Honolulu, HI, although some samples came from commercial farms throughout the state of Hawaii. In addition, samples were also used from archive materials collected from local and South Pacific kava growers and distributors between 1999 and 2003 and stored in dried, powdered form at −20 °C. Following harvest, the underground organs were washed free of soil and then cut into approximately 100 mm lengths.

Processing. Root samples ($n = 110$) were weighed using a Mettler P1012 top pan balance and then dried overnight in a Thelco blower oven at 70 °C. Percent moisture value was calculated on a dry weight basis. Dried samples were ground using a Wiley benchtop mill with a 2 mm screen. Particle size was not otherwise controlled or determined. Samples were then split into two subsamples, one subsample used for HPLC analysis and the other for NIRS analysis.

Extraction and HPLC Analysis. The method used for HPLC analysis is that described by Shao et al. (*11*) and is the current standard (*15*). Samples of the ground material (0.5−3.0 g) were suspended in 180 mL of methanol and homogenized for 1 min using a Tekmar tissue homogenizer (Tekmar Co., Cincinnati, OH). Kavalactones were extracted overnight by shaking on a Junior Orbit shaker (Lab-Line Instruments). After filtration through a glass fiber filter, extracts were quantitatively transferred to a measuring flask and made up to 200 mL final volume with methanol. An aliquot was then filtered through a 450 nm nylon filter and a 10 μL sample analyzed via HPLC. Isocratic HPLC using a 250 × 4.6 mm i.d. YMC 5S Basic 5 μm reversed-phase column (Waters Corp., Milford, MA) at a constant 40 °C, with a Shimadzu LC-10AS pump, Shimadzu SPD-10A UV detector, set at 220 nm separated the kavalactones. Chromatograms were recorded and quantitated on a Shimadzu CR501 integrator. The mobile phase was 20% methanol, 20% acetonitrile, 59.9% water, and 0.1% acetic acid on a volume basis with a flow rate of 1 mL/min.

Known concentration analytical standards (Addipharma GMBH, Hamburg, Germany; desmethoxyyangonin 99.73% purity, dihydrokavain 100% purity, dihydromethysticin 100% purity, methysticin 98.71% purity, kavain 100% purity) were interspersed between samples to verify that no time-related changes affected the HPLC results. Final percent dry weight content of each kavalactone was calculated from standard curves obtained from the individual analytical standards.

Near-Infrared Spectrometry. A NIRS 6500 instrument using NIRS3 software (NIRSystem Inc., Silver Spring, MD) was used to scan 22 mm ring cups in the linear transport attachment. Ring cups were filled with dry, ground kava samples and measured in diffuse reflectance. Five scans of each sample were made with approximately 72° rotation between each scan.

Calibration was carried out by partial least-squares regression (PLS) of absorption spectra expressed as log 1/Reflectance. Using the values obtained with HPLC as the analyte value, a separate calibration was made for each of the six kavalactones and for total kavalactones. Thus, a total of seven calibration equations were developed. PLS analysis was performed with NSAS software with 3:1 cross validation to prevent over fitting by the inclusion of excess factors (*16*).

Wavelength segments were selected using bifurcation searches to obtain equations with higher explanation of the total variability in the

Table 1. Minimum, Average, Maximum Individual Kavalactone Content of Calibration Set (% of dry weight)

	methy-sticin	yangonin	kavain	dihydro-methy-sticin	desmethoxy-yangonin	dihydro-kavain	total
minimum	0.09	0.08	0.11	0.08	0.08	0.10	0.54
average	1.37	1.41	1.98	0.99	0.84	1.27	7.86
maximum	2.70	3.02	3.92	2.58	2.35	3.33	14.68

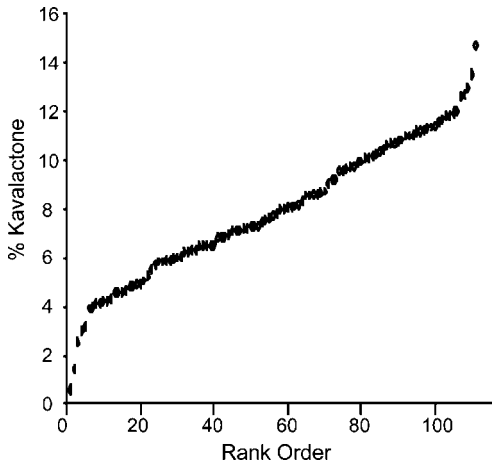

Figure 2. Plot of total kavalactones (% of dry weight) of calibration samples arranged in rank order.

calibration values (measured by R^2; multiple correlation coefficient) without increasing the number of PLS factors used. The algorithm included the following numbered steps. (1) The search was initiated by calibrating using all the wavelengths as the current segment. (2) Then the current segment was divided in two and three calibrations made, one each for the current segment and each half of the current segment each combined with previous segments (defined below). (3) The segment—of the three—with the best equation so far was chosen as the new current segment. (4) This was repeated from step 2 until the R^2 for the equation no longer improved without an increase in factors, i.e., the current segment did not change. (5) The halves of the two segments previously dropped that abut each end of the current segment were then added back to make a continuous new current segment. (6) A calibration was made with the current segment and the previous accepted segments. (7) The algorithm was repeated from step 2 until the equation no longer improved. (8) The current segment from step 7 was added to the set of previous segments. (9) The new current segment was chosen from those segments previously dropped, the criteria being the highest R^2 and the segment did not abut any previous segments used in the equation. (10) A calibration was made with this new current segment and the previous accepted segments. (11) Iterations were made from step 2 until the equation no longer improved.

The seven equations, one each for the six major kavalactones and total kavalactones, were validated using 12 samples collected for validation tests in addition to the internal cross validation of PLS. Each sample was presented five times for scanning and sampled three times for HPLC analysis. Predictions using the equations imported into the NIRS3 version 4.0 software (ISI Software, Inc.) were compared to the HPLC results.

RESULTS AND DISCUSSION

The calibration set of 110 samples covered the expected values of all six major kavalactones and total kavalactones in dried plant material (**Table 1**). When plotted in rank order, all six measured kavalactone concentrations were quite evenly spaced except at the high and low extremes. The plot of total kavalactones in rank order (**Figure 2**) has the typical shape for plots of all six individual kavalactones when plotted in rank order. The rank order was not the same for each of the measured kavalactone values, i.e., the individual kavalactones were independent of each other with six degrees of freedom. Obviously knowing six of the values allows calculation of the seventh.

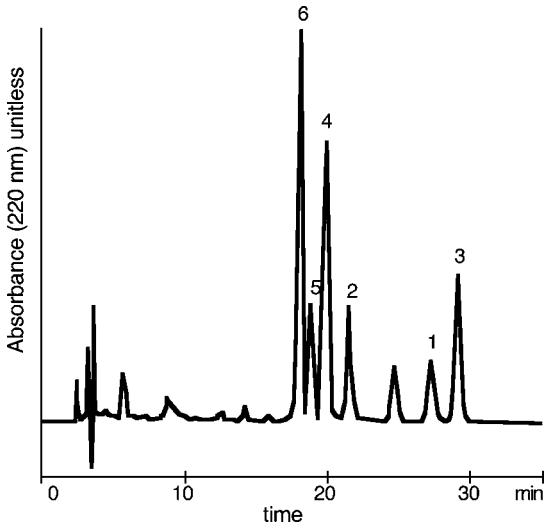

Figure 3. HPLC separation of six major kavalactones in kava underground tissues listed in order of elution: (6) methysticin, (5) dihydromethysticin, (4) kavain, (2) dihydrokavain, (1) desmethoxyyangonin, and (3) yangonin. Identifiers are chemotype numbers.

Although the samples were not selected or collected in the order plotted, **Figure 2** indicates the calibration set is evenly distributed over the range of expected values. Thus, the equations developed are representative of the full range of concentrations expected in dry underground plant organs of kava. Application of the equations to other plant organs or tissues or to concentrated extracts is not recommended; however, the evidence below indicates equations could be developed for kavalactones in other plant parts and extracts. In fact Gaub et al. (*14*) developed calibration equations using NIRS on total kavalactones and kavain in dry extracts of kava.

Use of the UV detector at 220 nm resulted in a chromatogram with less noise from interfering but uninteresting compounds than if set at 240 nm, confirming the findings of Shao (*11*). In addition, Shao (*11*) pointed out that while kavain, dihydromethysticin, and dihydrokavain have absorption maxima around 240 nm, methysticin, desmethoxyyangonin, and yangonin have absorption maxima around 220 nm. As most of the kava root samples used in this study have a considerably higher kavain content than the other kavalactones, use of 220 nm was preferred because this increased the detection sensitivity of the other kavalactones, particularly the three with absorption maxima at 220 nm. All standard curves were found to be linear. A typical chromatogram is shown as **Figure 3**.

PLS was applied to the kava sample spectra and the corresponding HPLC value. The HPLC value was assumed to be the actual value of the analyte for calibration. A typical spectrum is shown in **Figure 4**.

The equations obtained from the calibrations all have R^2 values that indicate the majority of the variation is explained by the respective equation (**Table 2**). The R^2 value is the highest for total kavalactones with 91% of the variation in the values explained. The equation for methysticin explains the least amount of variation: 59%. The two kavalactones, dihydrokavain and dihydromethysticin, without a double bond between carbons 4 and 5 and also between carbons 7 and 8 have high R^2 values: 0.86 and 0.88, respectively. Kavain, with a double bond between

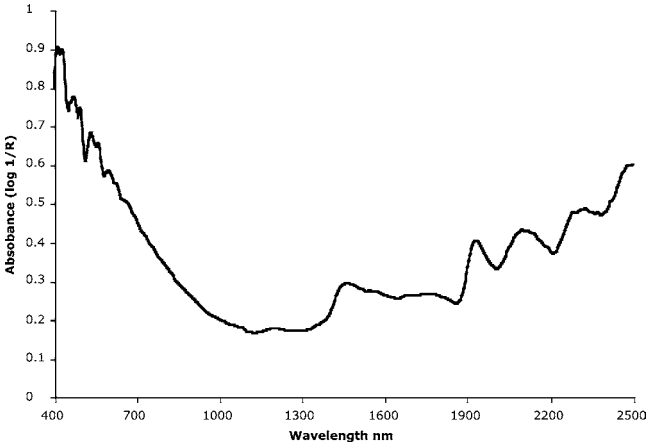

Figure 4. Typical spectrum showing diffusion reflectance (R) of dried, ground kava underground tissues over 400–2500 nm wavelengths.

carbons 7 and 8, has a lower R^2 value: 0.77. Adding a second double bond between carbons 4 and 5 lowers the R^2 value even more to 0.67 for desmethoxyyangonin and 0.62 for yangonin.

The double bonds at carbons 7 and 8 and 4 and 5 add noise to the spectra that cannot be modeled. Interestingly, the most noise comes from the combination of the $O-CH_2-O$ group on the benzene ring and a double bond between carbons 7 and 8 as in methysticin. This makes generalization difficult without more study.

The spectrum bands selected formed a pattern similar to that of the double bond between carbons 7 and 8. The exception is yangonin. All equations use the 1650−1750 nm band and either 2200−2300 or 2300−2400 nm band. PLS selects the higher energy band when the 7−8 double bond is absent and the lower energy band when that bond is present except for yangonin. Again, generalization is difficult without further study.

Unsurprisingly, the equation for the total kavalactones contains all three isolated bands of the spectrum.

Interferences may not have been completely modeled by PLS nor eliminated by the spectrum band selection. The R^2 values indicate a rather large portion of the variation was not modeled. Also, the standard errors from external validation are two to six times the standard error of observation of the particular kavalactone (including total) being modeled.

The water absorption bands are not part of the bands selected for the equations. This implies that equations for fresh (not dried) underground tissue are feasible. The information on kavalactone content would most likely not be obscured by the presence of water.

Because the samples were all ground with the same mill and screen size, particle size was most likely not part of the information in the spectra. That particle size affects the NIR reflectance is well known (*9*). Use of the equations reported here with other milling procedures would require extreme caution. Validation would be necessary with the new milling procedures with new calibrations made if validation errors are too large for acceptable predictions.

The standard errors from external validation are small enough for the equations to be of practical value (**Table 2**) in establishing the concentration of any individual kavalactone and the total kavalactones. The chemotype is determined from the concentrations. The standard errors of validation when normalized by division with the full-scale value of each kavalactone are on the order of 10%. The normalized error of external validation is least for methysticin, 6%, increasing for dihydromethysticin, 6%, total, 7%, desmethoxyyangonin, 7%, kavain, 8%, and dihydrokavain, 10%, to a maximum for yangonin, 12%.

Figure 5 shows the relationship between the values measured using NIRS and those measured with HPLC. Each cross depicts the mean and one standard deviation each side of the mean for both measurements. The line in the graph indicates a perfect mapping of NIRS and HPLC values. In no validation set have all measurements been within two standard errors of observation of the perfect line; however, the measurements are predominately 80% or more in this region.

Both NIRS and HPLC methods are highly reproducible (using the same sample presented in the same way the results are nearly identical). The differences between NIRS and HPLC are most likely caused by sampling and presentation. The results indicate the repeatability of both methods are approximately equal and therefore have similar accuracy if properly calibrated. Coupled with the overwhelming number of samples being within the error of repeatability (two standard deviations) of a perfect correlation, the evidence supports the use of NIRS for measuring kavalactones in dry powder made directly from underground kava organs. The study results are encouraging for development of other calibrations for quality control in kava processing even though the reported calibrations are only applicable to unspecified mixtures of underground organs of the kava plant that have been dried and ground.

The combination of near-infrared reflectance spectroscopy (NIRS) and partial least-squares (PLS) methods has been found to be a convenient, versatile, and rapid analytical tool for kavalactones determination in dried kava powder made directly from underground plant organs. Application of NIRS to routine evaluation of kavalactone content would eliminate the use of solvents except during quality control of the calibration. Preparation of a sample for NIRS requires only drying and grinding of the sample. The presentation and analysis of a sample takes only a few minutes with NIRS.

Table 2. Wavelengths Used for Kavalactone Calibration Equations and Final Performance Statistics

				standard error		
					observation	
constituent	wavelengths, nm	PLS factors	R^2	validation	NIRS	HPLC
desmethoxyyangonin	1650–1750 and 2300–2400	6	0.67	0.20	0.12	0.08
dihydrokavain	1650–1750 and 2200–2300	6	0.86	0.31	0.04	0.06
yangonin	1650–1750 and 2200–2300	5	0.62	0.47	0.05	0.10
kavain	1650–1750 and 2300–2400	5	0.77	0.21	0.11	0.08
dihydromethysticin	1650–1750 and 2200–2300	7	0.88	0.15	0.03	0.04
methysticin	1650–1750 and 2300–2400	5	0.59	0.19	0.06	0.06
total kavalactones	1650–1750 and 2200–2400	6	0.91	1.05	0.25	0.25

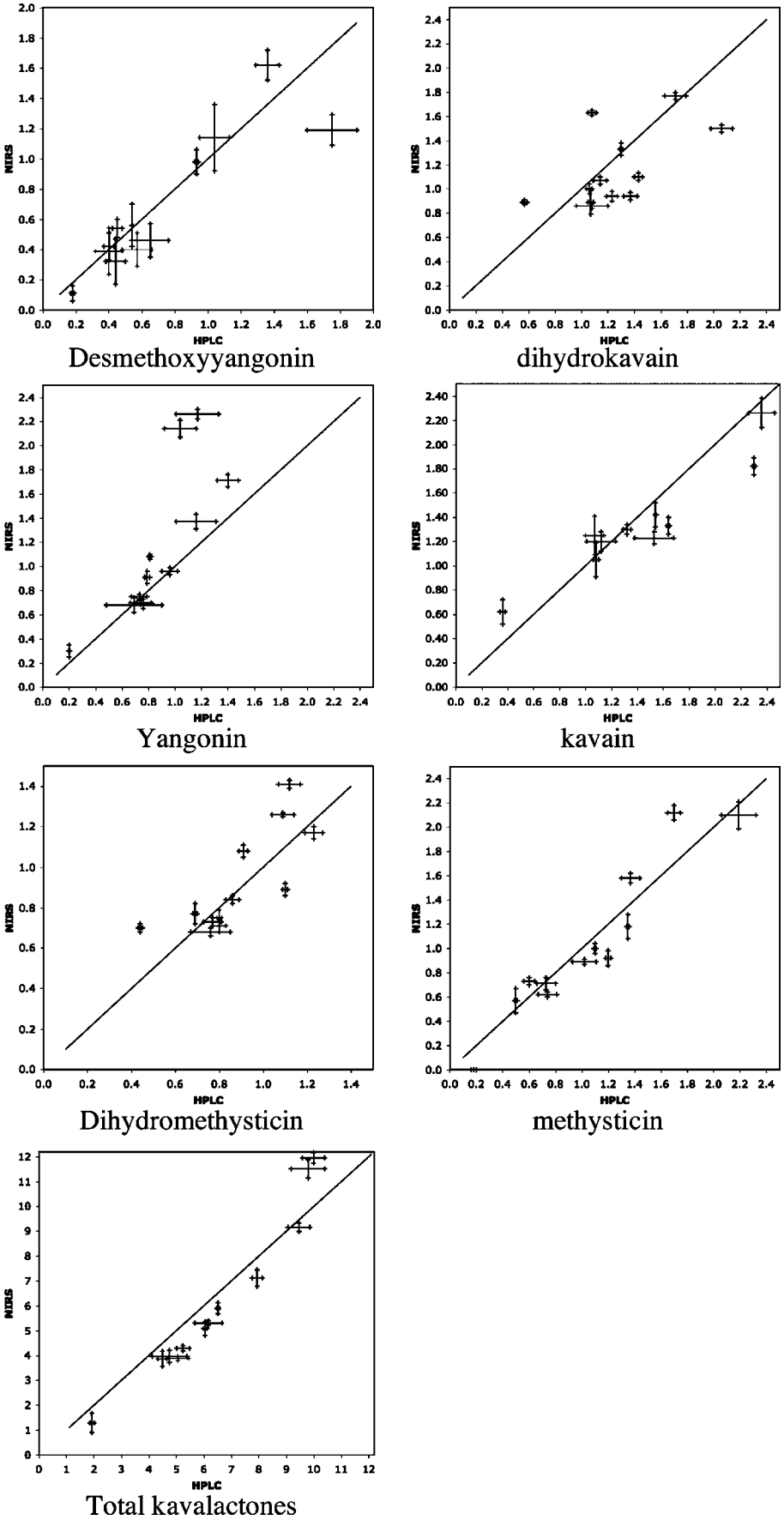

Figure 5. Best NIRS equation determination versus determination with HPLC of percent mass for (A) desmethoxyyangonin, (B) dihydrokavain, (C) yangonin, (D) kavain, (E) dihydromethysticin, (F) methysticin, and (G) total kavalactones. Crosses show one standard error for the corresponding method. Shade change in crosses is only to clarify which error belongs to a specific sample. The line indicates a perfect mapping of the NIRS and HPLC values.

ACKNOWLEDGMENT

The Agricultural Diagnostic Service Center of the College of Tropical Agriculture and Human Resources, University of Hawaii—Manoa, provided access to the NIR spectrometer for all scans.

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Received for review April 6, 2006. Revised manuscript received June 8, 2006. Accepted June 12, 2006. This project was funded by a USDA Tropical and Subtropical Research grant 00-34135-9576.

JF060964V