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Single-Lab Validation for Determination of Kavalactones and Flavokavains in *Piper methysticum* (Kava)

Authors
Ying Liu[1], Jensen A. Lund[2], Susan J. Murch[2], Paula N. Brown[1]

Affiliations
1 Natural Health and Food Products Research Groups, BC Institute of Technology
2 Department of Chemistry, University of British Columbia

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Correspondence
Dr. Paula N Brown
Centre for Applied Research and Innovation, British Columbia Institute of Technology
3700 Willingdon Ave, Burnaby, BC – V5G 3H2, Canada
Phone: + 1 604 412 7484, Fax: + 1 604 433 5893
paula_brown@bcit.ca

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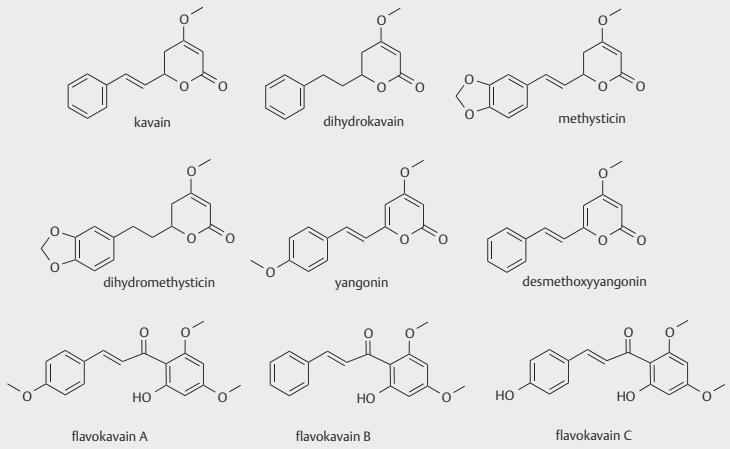
ABSTRACT

Piper methysticum (Kava) is a plant whose roots are used in the preparation of traditional beverages with spiritual, medicinal, and social importance for the Pacific Islanders. Kava is also sold as a herbal supplement or recreational beverage consumed for its mild inebriating effect in Europe and North America. With an ongoing interest in the safety and quality of kava products, it is necessary to develop a validated method for determination of kava chemical composition to ensure confidence in quality assessment. Thus, an high-performance liquid chromatography with ultraviolet detection (HPLC-UV) method was developed, optimized, and validated for determining six major kavalactones and three flavokavains in kava raw materials and finished products based on AOAC single-laboratory validation guidelines. This is the first fully validated analytical method for measuring kavalactones and flavokavains in a single run. The separation of the analytes was achieved in 10 min with an Agilent Poroshell C18 column using gradient separation. The sample was extracted with methanol first and then acetone. The signals were detected at 240 nm and 355 nm. The limit of quantification was under 1.2 µg/mL (0.3 mg/g) for kavalactones and under 0.35 µg/mL (0.01 mg/g) for flavokavains. The Horwitz ratio values described ranged from 0.3 to 1.82. The spike recovery experiments showed an accuracy between 92 and 105 % for all analytes. The results of the study demonstrate that the method is fit for the purpose of determining methysticin, dihydromethysticin, kavain, dihydrokavain, yangonin, desmethoxyyangonin, flavokavain A, flavokavain B, and flavokavain C in kava raw material and finished products (dry-filled capsule, liquid phytocaps, and tincture).

Introduction

Kava (*Piper methysticum* Forst. F.; family: Piperaceae) is a widely distributed plant across the south Pacific region [1]. There has been a long history of consuming kava beverage made from kava root/rhizome for spiritual, sociopolitical, ceremonial and medicinal purposes in the region [2, 3]. In the last century, kava has gained popularity in the Western world as a herbal supplement, primarily used to treat anxiety symptoms, and as a drink consumed recreationally for its mild inebriating effect [4, 5]. In the 1990 s, Germany approved kava for treating anxiety and nervous disorders; however, in 2001, several cases of acute liver toxicity were linked to kava use, raising concerns of kava safety and resulting in issuance of a U. S. Food and Drug Administration Consumer Advisory Warning on kava consumption the following year [6, 7]. In the same year, the European Union issued a temporary ban on imports of kava products [2, 8, 9]. The ban on kava caused an estimated damage of USD $1.2 billion to the European industry [6]. In 2007, the World Health Organization (WHO) published a report summarizing that kava is efficient at relieving anxiety, is relatively free of side effects, and is not addictive based on 16 controlled, double-blind studies and a meta-analysis of 11 high-quality clini-



▶ **Fig. 1** Chemical structure of kavalactones and flavokavains analyzed in this study.

cal trials [2, 7]. In 2015, the German kava ban was lifted by court [10]. Kava has regained its legal status in most countries; however, its trade, consumption, and distribution are strictly regulated and monitored [2]. A comprehensive review jointly published by the WHO and Food and Agriculture Organization on kava safety concluded that it is possible for kava beverage to be consumed with an acceptably low health risk. The report emphasized the need for developing practical and reliable analytical techniques that are capable of monitoring the active chemical ingredients as well as ingredients that may cause potential toxicity in kava for safety and quality concerns [3].

The quality of kava and kava products is usually determined and evaluated by the content of six most abundant kavalactones: kavain, dihydrokavain, methysticin, dihydromethysticin, yangonin, and desmethoxyyangonin. It was found that kava cultivars that are rich in kavain can produce a sudden relaxing effect, while cultivars high in dihydromethysticin and dihydrokavain can cause nausea [6]. Existing analytical methods for determining kavalactones include the usage of HPLC and/or liquid chromatography-mass spectrometry, gas chromatography, thin-layer chromatography, capillary zone electrophoresis, and near infrared reflectance spectroscopy [11, 12]. Among these methods, a reversed-phase HPLC method usually provides good separation and high accuracy; however, the long analysis time makes it unsuitable for routine analysis [11, 13]. Current HPLC methods for analyzing kavalactones have a run time of 30–50 min [13]. To the best of our knowledge, none of the methods for measuring kavalactones in the literature were fully validated.

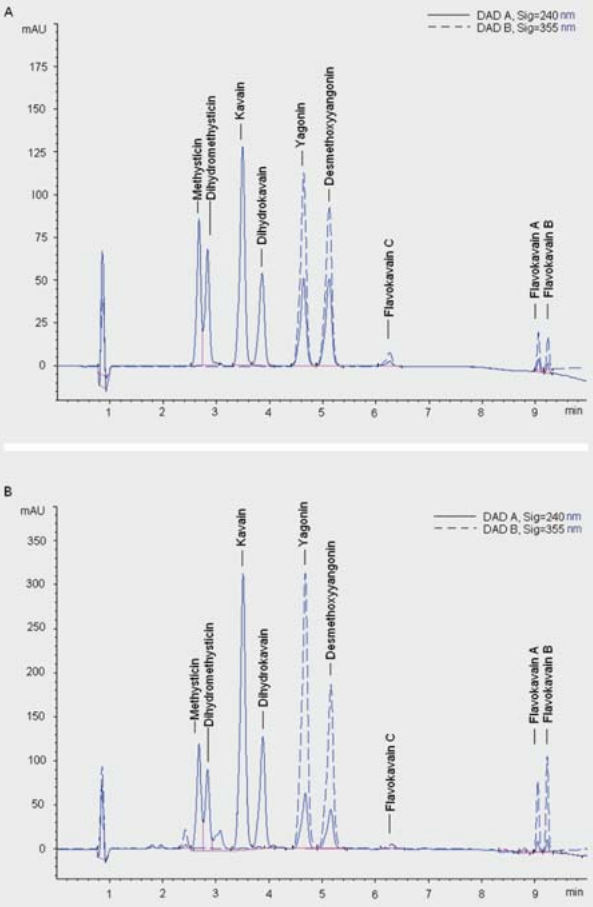
Recently, another class of chemicals in kava, flavokavains (including 3 compounds: flavokavains A, B, and C), has drawn researchers' attention due to their potential risks and benefits. Flavokavain B, in particular, is implicated in causing hepatotoxicity [13–15]. Flavokavain A may have shown anticarcinogenic properties based on mice models [6, 16]. The available methods for detecting flavokavains are limited in number. The latest HPLC method proposed by Meissner and Häberlein [13] for quantifying flavokavains along with kavalactones had a run time of 50 min. More efficient and validated analytical methods are needed to determine the content of both kavalactones and flavokavains for kava quality assessments.

In the present paper, we describe an HPLC protocol for rapid quantification (10 min) of six kavalactones and three flavokavains (▶ **Fig. 1**), simultaneously, in kava raw material and finished products. The HPLC method was fully validated based on AOAC International Guidelines for Single-Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals [17]. This AOAC document provides instructions specifically to validate methods for botanicals and dietary supplements, which have complex matrices [17]. After validation, the HPLC method studied can serve as a quality control tool in routine analysis of kava raw material and finished products.

Results and Discussion

Identification of analytes in the test materials was determined by comparing peak retention times and UV profiles to the reference standards. All six major kavalactones and three flavokavains were eluted separately following this order: methysticin, dihydromethysticin, kavain, dihydrokavain, yangonin, desmethoxyyangonin, flavokavain C, flavokavain A, and flavokavain B. Representative chromatograms of a mixed standards solution (▶ **Fig. 2 A**) and a test sample (▶ **Fig. 2 B**) are shown. An extended eight-point calibration curve range of 5–250 µg/mL (or 1.25–62.5 mg/g) was used for demonstration the linearity of the six kavalactones and an eight-point calibration range of 0.5–25 µg/mL (or 0.125–6.25 mg/g) was used for the three flavokavains. The R^2 value for all nine reference standard curves each day of analysis was ≥ 0.999 (**Fig. 1S**, Supporting Information).

The variance checks showed that the method used was applicable for the determination of nine analytes. The method detection limit (MDL) and limit of quantification (LOQ) for each analyte was calculated and reported in ▶ **Table 1**. The precision data indicated that all nine analytes had adequate precision in each of the four matrices (▶ **Table 2**). The repeatability relative standard devi-



▶ **Fig. 2** Representative chromatograms of a mixed calibration standard containing six kavalactones and three flavokavains (**A**) and a test sample (**B**) using current HPLC-UV method. Solid line represents UV absorbance at 240 nm, and dotted line represents UV absorbance at 355 nm.

ations (RSD_r) ranged from 9.06% to 0.95% for kavalactones and 12.99% to 2.44% for flavokavains, which are within the AOAC range for the sample concentration. Horwitz ratio (HorRat) values are used to evaluate method performance based on the ratios of actual precision to predicted precision. AOAC guidelines for single-laboratory validation accept a HorRat range from 0.5 to 2. In our method, the HorRat value for kavalactones ranged from 0.3 to 1.82 and for flavokavains ranged from 0.54 to 1.68. A HorRat value lower than 0.5 was considered acceptable, considering the analysis was performed under tightly controlled conditions.

A spike recovery study based on spiking six kavalactones and three flavokavains into a negative control material (goldenseal) at high, medium, and low levels were completed and shown in ▶ **Table 3**. Goldenseal root was chosen as a blank matrix or a negative control material because there is no kavalactones and flavokavains found in goldenseal. The average recovery for each analyte at each level were within the AOAC guidelines. AOAC guidelines for single-laboratory validation accept recovery limits from 92 to 105% at 1% concentration, 90 to 108% at 0.1% concentration, and 85 to 110% for 0.01 at 0.01% concentration.

With the ongoing interest in the safety and quality of kava products, it is necessary to develop a method that is validated to ensure confidence in analytical data. The HPLC-UV method described herein for the determination of six major kavalactones and three flavokavains in kava (*P. methysticum*) raw and select finished products was validated based on AOAC Guidelines for Single-Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals. This is the first fully validated analytical method for measuring kavalactones and flavokavains in kava with a rapid analysis time of 10 min. The results of the study demonstrate that the method is fit for the purpose of determining methysticin, dihydromethysticin, kavain, dihydrokavain, yangonin, desmethoxyyangonin, flavokavain A, flavokavain B, and flavokavain C in kava raw material and finished products (dry-filled capsule, liquid phytocaps, and tincture). Further collaborative study is recommended for evaluating reproducibility and a composite measure of variation that includes between-laboratory and within-laboratory variation. Kava commercial product information and calibration curve data, retention time data, and resolution data of kavalactones and flavokavains are available as Supporting Information.

▶ **Table 1** MDL and LOQ of kavalactones and flavokavains.

Analyte	MDL (µg/mL)	MDL (mg/g)	LOQ (µg/mL)	LOQ (mg/g)
Methysticin	0.27	0.07	0.94	0.23
Dihydromethysticin	0.19	0.05	0.66	0.16
Kavain	0.15	0.04	0.53	0.13
Dihydrokavain	0.16	0.04	0.54	0.13
Yangonin	0.24	0.06	0.84	0.21
Desmethoxyyangonin	0.35	0.09	1.20	0.30
Flavokavain C	0.06	0.02	0.22	0.06
Flavokavain A	0.10	0.03	0.35	0.09
Flavokavain B	0.07	0.02	0.26	0.06

▶ **Table 2** Precision summary of HPLC-UV method for detecting kavalactones and flavokavains in kava products.

Analyte	Matrix	Concentration	Unit	Intermediate precision	Overall RSD_r (%)	HorRat
Methysticin	Raw root material	10.51	mg/g	2.40	5.67	1.43
	Dry-filled capsule	21.59	mg/g	1.17	2.08	0.61
	Liquid phytocaps	8.73	mg/g	0.89	1.88	0.47
	Liquid tincture	2.32	mg/mL	5.19	4.88	0.98
Dihydromethysticin	Raw root material	7.69	mg/g	1.60	4.84	1.16
	Dry-filled capsule	19.02	mg/g	1.60	2.35	0.67
	Liquid phytocaps	8.16	mg/g	0.94	3.12	0.78
	Liquid tincture	2.58	mg/mL	5.10	6.25	1.27
Kavain	Raw root material	11.05	mg/g	1.74	4.93	1.25
	Dry-filled capsule	36.69	mg/g	1.15	3.36	1.06
	Liquid phytocaps	19.81	mg/g	0.86	5.26	1.49
	Liquid tincture	5.14	mg/mL	5.30	7.63	1.73
Dihydrokavain	Raw root material	10.31	mg/g	1.52	4.09	1.03
	Dry-filled capsule	35.94	mg/g	1.01	0.95	0.30
	Liquid phytocaps	18.17	mg/g	0.84	3.43	0.96
	Liquid tincture	5.71	mg/mL	5.32	6.20	1.42
Yangonin	Raw root material	7.49	mg/g	2.08	5.64	1.35
	Dry-filled capsule	18.47	mg/g	1.07	1.74	0.50
	Liquid phytocaps	8.41	mg/g	1.00	5.07	1.27
	Liquid tincture	1.11	mg/mL	2.62	5.83	1.05
Desmethoxyyangonin	Raw root material	3.00	mg/g	7.49	5.94	1.24
	Dry-filled capsule	12.80	mg/g	1.03	1.25	0.34
	Liquid phytocaps	6.65	mg/g	0.81	7.54	1.82
	Liquid tincture	2.01	mg/mL	5.30	9.06	1.78
Flavokavain C	Raw root material	0.13	mg/g	2.28	12.99	1.68
	Dry-filled capsule	0.40	mg/g	1.44	3.98	0.64
	Liquid phytocaps	0.17	mg/g	1.59	6.19	0.86
	Liquid tincture	< LOQ				
Flavokavain A	Raw root material	0.43	mg/g	1.51	5.96	0.93
	Dry-filled capsule	2.33	mg/g	1.07	2.65	0.55
	Liquid phytocaps	0.98	mg/g	0.76	3.18	0.58
	Liquid tincture	0.083	mg/mL	3.70	6.22	0.76
Flavokavain B	Raw root material	0.30	mg/g	1.60	7.64	1.13
	Dry-filled capsule	3.55	mg/g	1.07	2.44	0.54
	Liquid phytocaps	1.62	mg/g	0.85	3.30	0.64
	Liquid tincture	0.057	mg/mL	1.86	5.13	0.59

Materials and Methods

Reagents

Acetone (lab-grade, Anachemia), methanol (HPLC-grade, Anachemia), acetonitrile (HPLC-grade, Anachemia), 2-propanol (HPLC-grade, BDH), formic acid (88%, certified ACS, Fisher Scientific) were obtained from VWR International (Edmonton, AB, CAN). Water was purified to 18 MΩ using a Barnstead Smart2Pure nanopure system (Themo Scientific). Reference materials of D, L-kavain (purity: 95% HPLC), yangonin (purity: 95% HPLC), flavokavain A (purity: 95% HPLC), flavokavain B (purity: 95% HPLC), and flavokavain C (purity: 95% HPLC) were purchased from Extrasynthese. Reference materials of methysticin (purity: 98.98% HPLC), dihydromethysticin (purity 94.02% HPLC), desmethoxyyangonin (purity: 100% HPLC), and dihydrokavain (purity: 100% HPLC) were purchased from Cerilliant.

▶ **Table 3** Spike recovery results of HPLC-UV method for determination of kavalactones and flavokavains.

Analyte	High level (2.5%)	Medium level (1.25%)	Low level (0.5%)
Methysticin	92%	92%	94%
Dihydromethysticin	98%	97%	100%
Kavain	99%	92%	100%
Dihydrokavain	93%	92%	100%
Yangonin	98%	92%	97%
Desmethoxyyangonin	97%	93%	96%
Analyte	High level (0.25%)	Medium level (0.125%)	Low level (0.05%)
Flavokavain C	92%	94%	105%
Flavokavain A	92%	94%	104%
Flavokavain B	92%	94%	105%

Test materials and sample preparation

Kava commercial products (dry-filled capsules, liquid phytocaps, and tincture) were purchased from a local Whole Foods market (**Table 1S**, Supporting Information). Kava dry root powder packages were purchased from Gaia Garden Herbal Dispensary (Vancouver, BC, CAN). Ground Goldenseal powdered root material grown, harvested and authenticated (lot 10150401) by Sleepy Hollow Farms (Dalton, GA, USA) was obtained from Chromadex (Irvine, CA, USA) (**Table 1S**, Supporting Information). All the samples were analyzed in triplicate unless stated otherwise.

Root powders

Two hundred milligrams of the root powered was first extracted with 25 mL methanol and shook for 30 min with a wrist action shaker at room temperature. Extracts were centrifuged at 4500 *g* for 5 min, and the supernatant was transferred to a 50 mL volumetric flask. The remaining residue was re-extracted with 25 mL acetone, following the same procedure. After 30 min shaking and 5 min centrifuging, the supernatant was transferred to the same 50 mL volumetric flask. The volumetric flask was filled to the mark with methanol in the end, and the extracts were filtered through a 3-mm syringe fitted with a 0.22-µm nylon filter (VWR) into an amber glass HPLC vial and ready for LC analysis.

Capsules and phytocaps

The content of the 20 capsules or phytocaps were combined and mixed thoroughly. Two hundred milligrams of the capsules or phytocaps content was extracted with methanol and acetone following the same procedure as above.

Tincture

Tincture products were mixed thoroughly and diluted with methanol through a serial dilution. First, the tincture products were diluted 1 : 10 with methanol by mixing 100 mL tincture products with 900 mL methanol. Then 100 mL of the 10 times diluted tincture was diluted again with 900 mL methanol. The final solution was filtered and ready for LC analysis.

Kavalactone and flavokavain analysis

An Agilent 1200 Rapid Resolution LC system equipped with a temperature-controlled autosampler, binary pump, and UV detector was used for the analysis of kavalactones and flavokavains. The samples (2 µL) were injected to an Agilent Infinity Lab Poroshell 120 SB-C18 column (3.0 × 75 mm, 2.7 micron) of 55 °C and separated using a gradient separation. Gradient elution was performed using 0.1% formic acid in water (solvent A) and mixture of isopropanol and acetonitrile (7 : 3, v/v) (solvent B) at a flow rate of 0.4 mL/min. The gradient program was as follows: 0 min, 70% solvent A; 5 min, 65% solvent A; 7 min 10%; 8–10 min, 0% solvent A. The UV absorbance was detected at 240 nm for methysticin, dihydromethysticin, kavain, and dihydrokavain and 355 nm for yangonin, desmethoxyyangonin, and flavokavains (A, B, and C).

Single-laboratory validation parameters

This method was validated following AOAC International guidelines [17] for conducing single-laboratory validation. For all the standards, 1000 µg/mL stock solutions were prepared by dissolving individual reference material in methanol in volumetric flasks. The stock solution for each standard was then diluted to appropriate concentration to establish retention time and combined at different concentration levels for external calibration. The reference materials were stored at − 20 °C for long-term storage.

Selectivity

Each individual reference standard was injected into the HPLC-UV to establish the selectivity of the method. The resolution for each reference standard was calculated. An Rs > 1 between closely eluting components was considered acceptable (**Table 2S**, Supporting Information).

Linearity

The linearity for each reference standard was determined by an eight-point standard calibration curves. The standard curve for six kavalactones ranged from 5 µg/mL to 250 µg/mL (5 µg/mL, 10 µg/mL, 20 µg/mL, 40 µg/mL, 80 µg/mL, 125 µg/mL, 180 µg/mL, and 250 µg/mL). The standard curve for three flavokavains ranged from 0.5 µg/mL to 25 µg/mL (0.5 µg/mL, 1 µg/mL, 2 µg/mL, 4 µg/mL, 8 µg/mL, 12.5 µg/mL, 18 µg/mL, and 25 µg/mL). A simple linear regression was used to calculate R^2 value, the slope, and y-intercept of each curve for each analyte. An $R^2 \geq 99.9\%$ was considered acceptable. Calibration curves were prepared for each compound in triplicate over several days.

MDL and LOQ

The limit of detection of the method was determined using MDL guidelines from the U.S. Environmental Protection Agency [18]. The MDL is defined as the minimum concentration of substance that can be measured and reported with 99% confidence that the analyte concentration is greater than zero. MDL was determined by analyzing seven replicates of a standard solution containing very low concentrations of each analyte and calculated as the sample standard deviation (SD) times t-statistic value at $\alpha = 0.01$ and n − 1 degrees of freedom. The LOQ was calculated as 10 times the sample SD of the replicates used to determine MDL.

Precision

Four independent replicates of same sample were prepared and analyzed on three separate days (n = 4 × 3). The within-day, between-day, overall precision, and HorRat values of the method for all nine target compounds were calculated based on AOAC guidelines for single-laboratory validation.

Recovery

Spike recovery experiments were performed at three levels (high: 100 µg/mL, medium: 50 µg/mL, low: 20 µg/mL) for kavalactones and three levels (high: 10 µg/mL, medium: 5 µg/mL, low: 2 µg/mL) for flavokavains. Powdered goldenseal root material was analyzed and no kavalactones were determined to be present. The goldenseal material was thus used as a blank matrix for the spike recovery experiments. The appropriate amount of reference standards was used to spike the blank matrix, followed by the extraction process. Due to the cost of reference standards, for high-level spike recovery experiments, 4 mg sample was extracted with 1 mL extraction solvent (500 µL methanol and 500 µL acetone). For medium- and low-level spike recovery experiments, 20 mg sample was extracted with 5 mL extraction solvent (2.5 mL methanol and 2.5 mL acetone). Three replicates were performed at each level and the mean recovery was calculated.

Data analysis

Individual kavalactones and flavokavains were quantified as mg/g and µg/mL in solid and liquid matrices. Microsoft Excel was used for calculations and statistical analysis of the validation data.

Supporting Information

Kava commercial product information and calibration curve data, retention time data, and resolution data of kavalactones and flavokavains are available as Supporting Information.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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