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Agenda item 3 **CRD2**

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STANDARD OPERATING PROCEDURE FOR THE DETERMINATION OF KAVALACTONES AND FLAVOKAVAINS IN FRESH AND DRIED KAVA PRODUCTS

(Prepared by Fiji)

1,0 Introduction

Piper methysticum G. Forst. (Piperaceae) rhizomes and roots are peeled, grinded, macerated in cold water, and pressed through a cloth strainer to prepare kava, a non-alcoholic beverage. The composition and quality of kava can be highly variable, depending on the age of the plant, the variety, and the part used to prepare the beverage: roots, rhizomes or basal stems. The six major kavalactones (KLs: yangonin = Y, dihydrokavain = DHK, desmethoxyyangonin = DMY, kavain = K, dihydromethysticin = DHM and methysticin = M) are responsible for the physiological effect and are usually quantified with HPLC. There is a second group of molecules is flavokavins (FKs: A, B, C). The chemical composition of the kava extract is strongly influenced by the extraction solvent and extraction technique. This procedure is based on analytical procedure using High Performance Thin Layer Chromatography (HPTLC) and UltraHigh Performance Liquid Chromatography (UHPLC). The HPTLC is a validated procedure for 174 varieties of kava and UHPLC analysis for 6 varieties of Kava from the South Pacific. The UHPLC analysis is representative of the genetically distinct groups of “Nobels”, “Tudei” and “Wichmannii” Kava varieties.

2.0 Materials and methodology

2.1 Preparation of Samples

Wash by hand under cold running water the kava roots and peeled rhizomes. Cut into small pieces the kava organs with a knife. Sun-dry the kava pieces for 3 days (similar to traditional practises). Ground the dried kava matter into powder using a Forplex F00 1218 hammer mill to achieve <2 mm particle size and pack into labelled zip-log plastic bags. Further ground the kava powder to very fine kava flour texture using a coffee grinder. Weigh the kava flour sample then dry in an oven at 60°C for 6 hours.

2.2 Preparation of Reference Standard

Six kavalactone and three flavokavain standards of analytical grade purchased from Sigma-Aldrich include methysticin (M), dihydromethysticin (DHM), kavain (KAV), dihydrokavain (DHK), yangonin (Y), desmethoyyangonin (DMY), flavokavain A (FKA), flavokavain B (FKB) and flavokavain C (FKC). Accurately weigh 1.0mg individually the pure kava standard powder into 1ml acetone and store in dark at 4°C if analysed later. Conduct peak purity tests for the kava standards using the UV Vis spectrophotometer and compare the UV spectra. Conduct standard linearity curve check by using the HPTLC plates. Apply different stock solutions (0.1, 0.2, 0.4, 0.6, 0.8, 1.0 µL) of the six KLs and three FKs scan at 240nm (for M, DHM, K, DHK) and scan at 355nm (for Y, DMY, FKA, FKB, FKC).

2.3 Sample extraction

Weigh 10g of kava powder, transfer to a clean 50ml polypropylene centrifuge tube and add 30ml acetone. Sonicate the tubes in a water bath for 30min then transfer to a centrifuge instrument and set at4500 rpm for 10min. Transfer the supernatant to a 9mm wide opening screw thread vial of 2ml amber glass. Stores vials in refrigerator at 4°C in dark till required for analysis.

2.4 Identification

2.4.1 High Performance Thin Layer Chromatography (HPTLC)

2.4.1.1 Chemicals and reagents for HPTLC analysis

Analytical grade solvent (acetone, dioxane, hexane and methanol).

Silica gel 60 F254 plates (dimension; 20 x 10cm) using Camag HPTLC system with an automatic TLC sampler (ATS 4) coupled to an automatic developing chamber (ADC 2) and a visualizer as well as a TLC Scanner 4 controlled with winCATS software.

2.4.1.2 Instrument conditions

Standards and sample solutions were applied as bands (length of 8 mm, 250 nL/s delivery speed, track distance 8.0 mm, and distance from the edge of 15 mm). The 10 mL mobile phase used to develop the plates was hexane:dioxane (8:2 v/v) with a migration distance of 80 mm at room temperature after 30 s of pre-drying and no tank saturation. Visual documentation of the plates was carried out at 254 nm and 366 nm. The plates were then scanned in reflectance mode at 240 nm (for M, DHM, K and DHK) and at 355 nm (for Y, DMY, FKA, FKB, FKC) with D2 and W lamp slit dimension 8.00 mm × 0.20 mm, scanning speed 20 mm/s, and data resolution 100 µm/step. Peak area measurements (in area units, AU) were used. The total analytical time was 50 min for 20 samples and 10 mL of mobile phase (corresponding to 2.5 min and 0.5 mL per sample).

2.4.2 UltraHigh Performance Liquid Chromatography (UHPLC)

2.4.2.1 Chemicals and reagents for UHPLC analysis

0.4% formic acid in water, acetonitrile (HPLC grade)

Ultra-high purity Nitrogen gas

2.4.2.2 Instrument conditions

UHPLC-DAD-ESI-MS/MS. (Q/TOF) analyses were performed on an Agilent Infinity® 1290 system (Agilent Technologies, Santa Clara, CA, USA) coupled to a UV/vis DAD detector and equipped with a QTOF 6530 detector (Agilent) controlled by MassHunter® software (version B.08.00, Agilent, Santa Clara, CA, USA). Analytical separation was carried out on a Poroshell® 120 EC-C18 column (100 mm × 3.0 mm, 2.7 µm) equipped with a pre-column (Poroshell® 20 EC-C18, 5 mm × 3.0 mm, 2.7 µm). A gradient of 0.4% formic acid in water (A) and acetonitrile (B) was used as follows: 0 min, 1% B; 1.0 min, 1% B; 6 min, 15% B; 12 min, 45% B; 14 min, 100% B; and 16 min, 100% B. The flow rate and column temperature were 0.9 mL min−1 and 60 ◦C, respectively. A total of 1.0 µL of sample extract was injected. The ESI source was optimized as follows for positive and negative ionization modes (in “Auto MSMS” acquisition mode): scan spectra from m/z 50 to 2000, capillary voltage 3.5 kV, nozzle voltage 2000 V, fragmentor 110 V, and fixed collision-induced dissociation (CID) energy at 20 eV. Nitrogen was used as the nebulizing gas with a flow rate of 12 L min−1 and a temperature of 310 ◦C at 40 psi. DAD was set at 240 nm and 355 nm. Peaks of the target KLs and FKs were spotted on the MS chromatograms based on their high resolution masses [26] and quantified by integrating manually peak areas at 240 nm (for M, DHM, K and DHK) or 355 nm (for Y, DMY, FKA, FKB, FKC).

3.0 References

Lebot, V., Michalet, S., Legendre, L. (2019). Kavalctone and Flavokavins Profile Conttribute to Quality Assessment of Kava (Piper methysticum G. Forst), the Traditional Beverage of the Pacific. *Beverages*. 2019, 1-14.