KRA-02

Method Title: Quantification of Mitragynine in Kratom Raw Materials and Finished Products by High-Performance Liquid Chromatography: Singly Laboratory Validation

Submitted by: Elizabeth Mudge

Submitted by Email: Elizabeth.mudge@bcit.ca

Enclosures: 1

Submitter notes:

We are planning on submitting this work for publication in J AOAC International. A manuscript will be submitted as soon as possible.

Quantification of Mitragynine in Kratom Raw Materials and Finished Products by High-Performance Liquid Chromatography: Single-Laboratory Validation

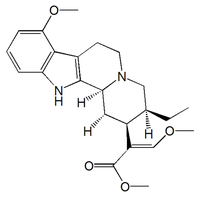
Elizabeth M. Mudge, Paula N. Brown
Natural Health & Food Products Research Group, BC Institute of Technology, Burnaby, British Columbia, Canada, V5G 3H2

BACKGROUND

Mitragyna speciosa Korth, also referred to as kratom or ketum, is a tropical tree indigenous to Southeast Asia. The leaves have traditionally been safely consumed as a stimulant to improve work performance, relieve aches and pains and has more recently been used for the treatment of diabetes and mitigation of opioid withdrawal (1, 2).

The major biologically active ingredient is the indole alkaloid mitragynine. It accounts for up to 66% of the total alkaloid content, while the other alkaloids present include: paynantheine, speciogynine, speciociliantine and 7-hydroxymitragynine (3).

Kratom was identified as a high priority ingredient by AOAC's SPDS Advisory Panel, where an SMPR[SM] has been generated. This method was developed for consideration as a First Action Official Method for the quantitation of mitragynine.



Structure of Mitragynine

MATERIALS

Eight test materials were sourced from commercial vendors.

Test Sample Code	Product Matrix	Contents
KT-RM01	Dried, ground leaf	Red vein powder
KT-RM02	Dried, ground leaf	White vein powder
KT-RM03	Dried, ground leaf	Maeng Da powder
KT-BE01	Dried, powdered, bulk extract	10:1 extract
KT-FP01	Ground, packaged product	Kratom
KT-FP02	Capsule	Maeng Da
KT-LB01	Liquid beverage	Kratom with a combination of proprietary herbs
KT-LB02	Liquid beverage	Kratom containing matcha, California poppy

Calibration standard for mitragynine was purchased from Chromadex and qualified using certified reference material from Cerilliant.

METHODS

<u>Method Development</u>

Extraction Solvent Selection: Evaluated extraction using Methanol, 70% methanol, 80% methanol, dichloromethane:methanl 50:50 %v/v and acetone.

Factorial Design: Evaluated 3 factors: solvent volume (10 mL, 20 mL), time (30, 60 min), and solvent composition (70% methanol, 70% methanol with 0.1 M acetic acid).

Chromatography: Several columns C8, C18, Synergi-hydro, C18 EVO (Phenomenex) were selected to optimize the separation of all alkaloids. The composition of the aqueous mobile phase was also adjusted from pH 4 to 9.5.

<u>Validation</u>

Sample Preparation: 100 mg of raw materials, capsules, bulk extracts and finished product ground material were extracted with 10 mL of 0.5 M acetic acid in 70% methanol for 1 hour by wrist action shaking. Liquid samples were diluted with methanol.

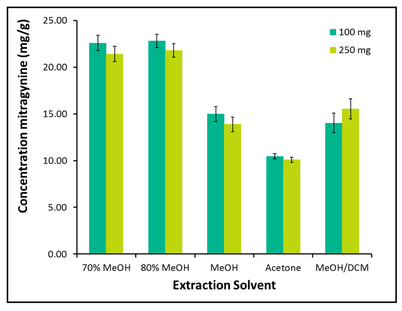
Photo Credit: Will McClatchey, 2015

HPLC Analysis: Gradient elution with 5 mM ammonium bicarbonate (pH 9.5) and acetonitrile using Phenomenex Kinetex C18 EVO 4.6 x 150 mm column. Run time: 18 minutes. Monitored at 226 nm.

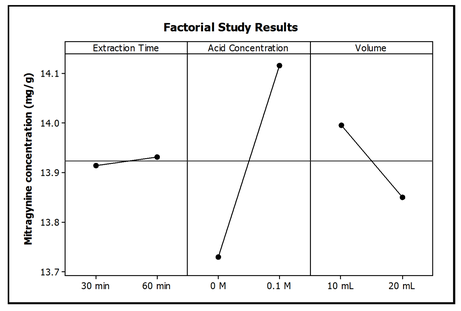
Performance Characteristics: Precision was determined by preparing four replicates of each test sample on three separate days. Seven replicates of a diluted extract were injected to determine the method detection limit (MDL) and limit of quantitation (LOQ) using the US EPA MDL protocol (4). Recovery was determined at three levels using a negative control sample.

RESULTS

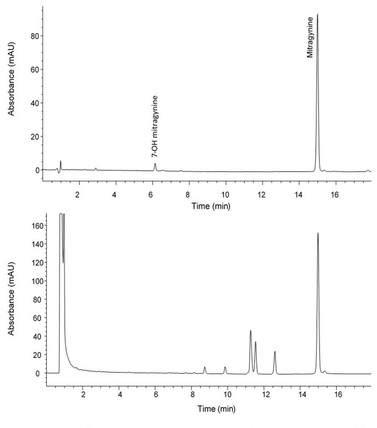
<u>Method Development</u>



Concentration of Mitragynine using different extraction solvents



Concentration of Mitragynine using Factorial Study



Chromatographic separation of Kratom alkaloids

Method development and optimization found that the addition of acid increased the extraction of the major alkaloids, which was further optimized to 0.5 M acetic acid with 70% methanol.

Chromatographic separation of all major alkaloids was optimal at pH 9.5, therefore the Phenomenex C18 EVO column was most suitable.

<u>Validation</u>

Concentration of Mitragynine using different test samples

Sample ID	Matrix	Mitragynine (mg/g)	RSD (%)	HorRat
KT-RM01	Dried, ground leaf	16.21	5.84	1.57
KT-RM02	Dried, ground leaf	15.89	7.32	1.96
KT-RM03	Dried, ground leaf	14.85	3.76	1.00
KT-BE01	Dried, powdered, bulk extract	68.52	3.87	1.29
KT-FP01	Ground, packaged product	12.20	5.38	1.39
KT-FP02	Capsule	11.90	5.03	1.29
		Mitragynine (µg/mL)		
KT-LB01	Liquid beverage	94.15	5.71	0.71
KT-LB02	Liquid beverage	379.45	4.47	0.68

Method detection limit and limit of quantitation

Analyte	MDL (µg/mL)	LOQ (µg/mL)
Mitragynine	0.2	0.6

Recovery of mitragynine using negative control sample

	Concentration (% w/w)		
Analyte	0.5	1.0	2.5
Mitragynine	105.2	106.0	100.9

CONCLUSION

The separation of several kratom alkaloids was possible with this method. The performance characteristics are within acceptable ranges according to AOAC International guidelines for dietary supplements (5). 7-OH mitragynine was below quantitation limit for all samples, therefore this method is only valid for the detection and quantitation of mitragynine in raw materials, bulk extracts and finished products.

REFERENCES

(1) Vicknasingam, B., Narayanan, S., Beng, G.T., Mansor, S.M. (2010) Int. J. Drug Policy, 21, 283-288. (2) Tanguay, P. (2011) Legislative Reform of Drug Policies, 13, 1-16. (3) Kikuri-Hanajiri, R., Kawamura, M., Maruyama, T., Kitajima, M., Takayama, H., Goda, Y. (2009) Forensic Toxicol. 27, 67-74. (4) Environmental Protection Agency (2002) Guidelines Establishing Test Procedures for the Analysis of Pollutants; Procedures for Detection and Quantification, 40 CFR pt. 136, Appendix D, rev. 1.11. Accessed September 11, 2015 from http://www.epa.gov/region6/qa/qadevtools/mod4references/analytical_references/40cfr136_03.pdf (5) AOAC International (2013). Appendix K: Guidelines for Dietary Supplements and Botanicals: Part I AOAC Guidelines for Single-Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals. Official Methods of Analysis. Gaithersburg, MD.

Kratom Method Details:

HPLC method conditions

Phenomenex Kinetex C18 EVO 150mm x 4.6mm, 5µm
Mobile Phase "A": 5 mM ammonium bicarbonate, pH 9.5
Mobile Phase "B": 100% Acetonitrile
The mobile phase is changed linearly according to the following program:

Time (min)	% Solvent A	% Solvent B
0.00	70	30
17.00	35	65
17.90	35	65
18.00	70	30

Flow rate: 1.5 mL/minute
Injection volume: 5 µL
Detection wavelength: 226 nm
Stop Time: 18.00min + 4 minutes post run (22.00 minutes)
Column Temperature: 25°C

Calibration standard solutions

Alkaloid	Approximate concentration (µg/mL)						
	Lin 1	Lin 2	Lin 3	Lin 4	Lin 5	Lin 6	Lin 7
Mitragynine	500	250	100	50	25	10	1
7-hydroxymitragynine	25	20	10	5	2	1	0.1

Sample Preparation Procedures

Raw Materials

1. For leaf material, grind to 0.75µm particle size in the centrifugal grinding mill.

2. On an analytical balance, weigh out approximately 100.0 ± 5.0 mg of powder or leaf material into separate, labelled eppendorf centrifuge tubes.

3. Add, using a volumetric pipette, 10.00 mL of extraction solvent to each tube.

4. Vortex each tube for 30 seconds.

5. Shake on wrist action shaker for 30 minutes.

6. Centrifuge for 5 minutes at 5000 rpm.

7. Filter a portion of sample through 0.22µm PTFE or nylon membrane filter into a glass HPLC vial, cap the vial and then analyze the sample using the HPLC method described below.

Bulk Extracts

1. On an analytical balance, weigh out approximately 100.0 ± 5.0 mg of powder or leaf material into separate, labelled eppendorf centrifuge tubes.

2. Add, using a volumetric pipette, 10.00 mL of extraction solvent to each tube.

3. Vortex each tube for 30 seconds.

4. Shake on wrist action shaker for 30 minutes.

5. Centrifuge for 5 minutes at 5000 rpm.

6. If concentration of alkaloids is higher than calibration data, a 1:5 dilution with extraction solvent is required. For these samples, dilute 200 µL of extract with 800 µL extraction solvent. Vortex for 30 seconds.

7. Filter a portion of sample through 0.22µm PTFE or nylon membrane filter into a glass HPLC vial, cap the vial and then analyze the sample using the HPLC method described below.

Finished Products (Capsules, Packets)

1. Combine the contents of 10 capsules, tablets, etc. Ensure sample is mixed for homogeneity (grind or stir contents to mix).

2. On an analytical balance, weigh out approximately 100.0 ± 5.0 mg of powder or leaf material into separate, labelled eppendorf centrifuge tubes.

3. Add, using a volumetric pipette, 10.00 mL of extraction solvent to each tube.

4. Vortex each tube for 30 seconds.

5. Shake on wrist action shaker for 30 minutes.

6. Centrifuge for 5 minutes at 5000 rpm.

7. Filter a portion of sample through 0.22µm PTFE or nylon membrane filter into a glass HPLC vial, cap the vial and then analyze the sample using the HPLC method described below.

Liquid Samples/Beverages

1. Shake bottle vigorously to disperse all contents of the solution.

2. For dilute samples (beverages): pipette 500 µL sample into a microcentrifuge tube. Add 500 µL methanol.

3. For concentrated samples (prototypes): pipette 100 µL sample into a microcentrifuge tube. Add 900 µL of methanol.

4. Vortex samples for 30 seconds.

5. Filter through a 0.22 µm Teflon or nylon filter into a glass HPLC vial. Analyse the sample using the HPLC method described below.